FORM PTO-1390 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE (REV. 1094) TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		ATTORNEY'S DOCKET NUMBER L0461/7102 U S APPLICATION NO (If known, see 37 CFR 1.5) 09/786214
INTERNATIONAL APPLICATION NO. PCT/US99/20344	INTERNATIONAL FILING DATE 03 September 1999 (03 09.99)	PRIORITY DATE CLAIMED 04 September 1998 (04.09.98)

TITLE OF INVENTION
AN ANTIGENIC PEPTIDE ENCODED BY AN ALTERNATIVE OPEN READING FRAME OF HUMAN MACROPHAGE COLONY-STIMULATING FACTOR

APPLICANT(S) FOR DO/EO/US
PROBST-KEPPER, Michael; VAN DEN EYNDE, Benoit; BOON-FALLEUR, Thierry

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U S C. 371.
3. ☒ This express request to begin national procedures (35 U S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US)

6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)) with verification of translation.

7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3))

9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(C)(4)).

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(C)(5)).

Items 11. To 16. Below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98 with references.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included

13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification (submitted as a first Preliminary Amendment).

15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or information:
    Copy of Page one of the PCT Published Application
    Copy of International Preliminary Examination Report

Express Mail Label No. EL310414711US Mailed March 1, 2001

528 Rec'd PCT/PTO 01 MAR 2001

U.S. APPLICATION NO (If known, see 37 C F R 1.5) 09/786214	INTERNATIONAL APPLICATION PCT/US99/20344	ATTORNEY'S DOCKET NUMBER L0461/7102

17. X The following fees are submitted:

CALCULATIONS PTO USE ONLY

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):

Search Report has been prepared by the EPO or JPO $860.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) $690.00

No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1 445(a)(2)).. $710.00

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1 445(a)(2)) paid to USPTO $1000.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) $100.00

ENTER APPROPRIATE BASIC FEE AMOUNT =				$690.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 X 30 months from the earliest claimed priority date (37 CFR 1.492(c)).				$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	22-20 =	2	X $18.00	$36.00	
Independent Claims	9- 3 =	6	X $80.00	$480.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+$270 00	$	
TOTAL OF ABOVE CALCULATIONS =				$1206.00	
Reduction by ½ for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1 28).				$	
SUBTOTAL =				$	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$	
Fee for recording the enclosed assignment (37 CFR 1 21(h)). The assignment must be accompanied by an appropriate coversheet (37 CFR 3.28, 3.31). $40 00 per property +				$	
TOTAL FEES ENCLOSED =				$1206.00	
				Amount to be refunded	$
				charged	$

a. ☒ A check in the amount of $ 1206.00 to cover the above fees is enclosed.

b. ☐ Please charge by Deposit Account No 23/2825 In the amount of $ _____ To cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 23/2825. A duplicate of this sheet is enclosed

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO

John R. Van Amsterdam
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John R. Van Amsterdam
SIGNATURE

John R Van Amsterdam
NAME

40,212
REGISTRATION NO

 #4

Attorney's Docket No: L0461/7102

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Probst-Kepper et al.
U.S. Serial No.	:	09/786,214
Int'l Application No.	:	PCT/US99/20344
Int'l Filing Date	:	03 September 1999 (03.09.99)
Earliest Priority Date	:	04 September 1998 (04.09.98)
Title	:	AN ANTIGENIC PEPTIDE ENCODED BY AN ALTERNATIVE OPEN READING FRAME OF HUMAN MACROPHAGE COLONY-STIMULATING FACTOR

CERTIFICATE OF MAILING UNDER 37 C.F.R. §1.8(a)

The undersigned hereby certifies that this document is being placed in the United States mail with first-class postage attached, addressed to Box PCT, Commissioner for Patents, Washington, D.C. 20231, on the 12th day of June, 2001.

Monica E. Zombori

Box PCT
Commissioner for Patents
Washington, DC 20231

STATEMENT PURSUANT TO 37 CFR 1.821(f) AND 37 CFR 1.825 (a) and (b)

This statement is made pursuant to 37 CFR 1.821 (f), and 37 CFR 1.825 (a) and (b). Applicants submit herewith a substitute copy of the computer readable diskette to comply with the sequence requirements.

Applicants' attorney states that the information recorded in computer readable form is identical to the written sequence listing which was filed with the PCT patent application on September 3, 1999. The replacement computer readable form contains no new matter.

Respectfully submitted,

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Attorney's Docket No.: L0461/7102
Date: June 12, 2001
x07/18/01

540792.1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	PROBST-KEPPER et al.
International Application No.	:	PCT/US99/10424
International Filing Date	:	03 September 1999 (03.09.99)
Earliest Priority Date	:	04 September 1998 (04.09.98)
Title	:	AN ANTIGENIC PEPTIDE ENCODED BY AN ALTERNATIVE OPEN READING FRAME OF HUMAN MACROPHAGE COLONY-STIMULATING FACTOR

Commissioner for Patents
Box PCT
Washington, DC 20231

PRELIMINARY AMENDMENT

Dear Sir:

Please amend the application as follows, prior to calculation of the fees.

In the Claims

Please cancel claims 4-6, 9, 10, 13-17, 20, 23-26, 29-32, 35-39, 42-44, 47, 50-57 and 60-64 without prejudice.

Please amend the claims as follows. Applicants have included herewith pages showing the markups of the claims with insertions and deletions indicated by underlining and bracketing, respectively.

11.(amended) An isolated nucleic acid encoding the immunogenic polypeptide of claim 2, wherein the isolated nucleic acid, when translated in frame, does not encode M-CSF, a precursor of M-CSF, or a fragment of M-CSF.

40.(amended) An isolated polypeptide which binds selectively the polypeptide of claim 2, provided that the isolated polypeptide is not an HLA class I molecule.

41.(amended) The isolated polypeptide of claim 40, wherein the isolated polypeptide is a monoclonal antibody, a chimeric antibody or a humanized antibody.

Remarks

Applicants have amended claim 40 to eliminate multiple dependencies. Claim 41 as amended contains the subject matter of claims 41-43 as filed. No new matter has been added.

Respectfully submitted,

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Attorney's Docket No. L0461/7102
Dated: March 1, 2001
X03/04/01

Amended Claims

11.(amended) An isolated nucleic acid encoding [a peptide selected from the group consisting of the polypeptide of claim 1 and] the immunogenic polypeptide of claim 2, wherein the isolated nucleic acid, when translated in frame, does not encode M-CSF, a precursor of M-CSF, or a fragment of M-CSF.

40.(amended) An isolated polypeptide which binds selectively [a] the polypeptide of [any of] claim[s 1,] 2, [3 or 4,] provided that the isolated polypeptide is not an HLA class I molecule.

41.(amended) The isolated polypeptide of claim 40, wherein the isolated polypeptide is [an] a monoclonal antibody, a chimeric antibody or a humanized antibody.

Attorney's Docket No: **L0461/7102**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Probst-Kepper et al.
U.S. Serial No.	:	09/786,214
Int'l Application No.	:	PCT/US99/20344
Int'l Filing Date	:	03 September 1999 (03.09.99)
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Title	:	AN ANTIGENIC PEPTIDE ENCODED BY AN ALTERNATIVE OPEN READING FRAME OF HUMAN MACROPHAGE COLONY-STIMULATING FACTOR

CERTIFICATE OF MAILING UNDER 37 C.F.R. §1.8(a)

The undersigned hereby certifies that this document is being placed in the United States mail with first-class postage attached, addressed to Box PCT, Commissioner for Patents, Washington, D.C. 20231, on the 12th day of June, 2001.

Monica E. Zombori

Box PCT
Commissioner for Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

Sir:

Please amend the United States national phase application of the above-identified PCT international application as follows.

In the Specification

Please add the following section as the first section of the specification following the title.

Related Applications

This application is a national stage filing under 35 U.S.C. § 371 of PCT/US99/20344, filed September 3, 1999, which was published under PCT Article 21(2) in English. This application claims the benefit under 35 U.S.C. §119(e) of United States provisional application 60/099,077, filed September 4, 1998.

Remarks

Applicants have amended the specification solely to provide priority application information and information regarding the publication in English under PCT Article 21(2) of the PCT application of which the above-identified application is a U.S. national stage application. No new matter has been added.

Respectfully submitted,

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Attorney's Docket No.: L0461/7102
Date: June 12, 2001
x07/18/01

Newly Added Section

Related Applications

This application is a national stage filing under 35 U.S.C. § 371 of PCT/US99/20344, filed September 3, 1999, which was published under PCT Article 21(2) in English. This application claims the benefit under 35 U.S.C. §119(e) of United States provisional application 60/099,077, filed September 4, 1998.

AN ANTIGENIC PEPTIDE ENCODED BY AN ALTERNATIVE OPEN READING FRAME OF HUMAN MACROPHAGE COLONY-STIMULATING FACTOR

Field of the Invention

This invention relates to polypeptides and encoded nucleic acid molecules which are expressed preferentially in tumors, particularly in renal cell carcinomas. The nucleic acid molecules and encoded polypeptides are useful in, *inter alia*, diagnostic and therapeutic contexts.

Background of the Invention

The phenotypic changes which distinguish a tumor cell from its normal counterpart are often the result of one or more changes to the genome of the cell. The genes which are expressed in tumor cells can be termed "tumor associated" genes. These tumor associated genes are markers for the tumor phenotype. The expression of tumor associated genes can also be an essential event in the process of tumorigenesis.

Typically, the host recognizes as foreign the tumor associated genes which are not expressed in normal non-tumorigenic cells. Thus, the expression of tumor associated genes can provoke an immune response against the tumor cells by the host. Tumor associated genes can also be expressed in normal cells within certain tissues without provoking an immune response. In such tissues, expression of the gene and/or presentation of an ordinarily immunologically recognizable fragment of the protein product on the cell surface may not provoke an immune response because the immune system does not "see" the cells inside these immunologically privileged tissues. Examples of immunologically privileged tissues include brain and testis.

The discovery of tumor associated expression of a gene provides a means of identifying a cell as a tumor cell. Diagnostic compounds can be based on the tumor associated gene, and used to determine the presence and location of tumor cells. Further, when the tumor associated gene contributes to an aspect of the tumor phenotype (e.g., unregulated growth or metastasis), the tumor associated gene can be used to provide therapeutics such as antisense nucleic acids which can reduce or substantially eliminate expression of that gene, thereby reducing or substantially eliminating the phenotypic aspect which depends on the expression of the particular tumor associated gene.

The process by which the mammalian immune system recognizes and reacts to foreign or alien materials is a complex one. An important facet of the system is the T cell response. This response requires that T cells recognize and interact with complexes of cell surface molecules, referred to as human leukocyte antigens ("HLA"), or major histocompatibility complexes ("MHCs"), and peptides. The peptides are derived from larger molecules which are processed by the cells which also present the HLA/MHC molecule. See in this regard Male et al., Advanced Immunology (J.P. Lipincott Company, 1987), especially chapters 6-10. The interaction of T cells and complexes of HLA/peptide is restricted, requiring a T cell specific for a particular combination of an HLA molecule and a peptide. If a specific T cell is not present, there is no T cell response even if its partner complex is present. Similarly, there is no response if the specific complex is absent, but the T cell is present. The mechanism is involved in the immune system's response to foreign materials, in autoimmune pathologies, and in responses to cellular abnormalities. Much work has focused on the mechanisms by which proteins are processed into the HLA binding peptides. See, in this regard, Barinaga, *Science* 257: 880, 1992; Fremont et al., *Science* 257: 919, 1992; Matsumura et al., *Science* 257: 927, 1992; Latron et al., *Science* 257: 964, 1992.

The mechanism by which T cells recognize cellular abnormalities has also been implicated in cancer. For example, in PCT application PCT/US92/04354, filed May 22, 1992, published on November 26, 1992, and incorporated by reference, a family of genes is disclosed, which are processed into peptides which, in turn, are expressed on cell surfaces, which can lead to lysis of the tumor cells by specific CTLs. The genes are said to code for "tumor rejection antigen precursors" or "TRAP" molecules, and the peptides derived therefrom are referred to as "tumor rejection antigens" or "TRAs". See Traversari et al., *J. Exp. Med.* 176:1453-1457, 1992; van der Bruggen et al., *Science* 254: 1643,1991; De Plaen et al., *Immunogenetics* 40:360-369, 1994 and U.S. Patent No. 5,342,774 for further information on this family of genes.

In U.S. Patent No. 5,405,940, the disclosure of which is incorporated by reference, nonapeptides are taught which are presented by the HLA-A1 molecule. The reference teaches that given the known specificity of particular peptides for particular HLA molecules, one should expect a particular peptide to bind one HLA molecule, but not others. This is important, because different individuals possess different HLA phenotypes. As a result, while identification of a particular peptide as being a partner for a specific HLA molecule has

diagnostic and therapeutic ramifications, these are only relevant for individuals with that particular HLA phenotype. There is a need for further work in the area, because cellular abnormalities are not restricted to one particular HLA phenotype, and targeted therapy requires some knowledge of the phenotype of the abnormal cells at issue.

In U.S. Patent 5,629,166, incorporated by reference, the fact that the MAGE-1 expression product is processed to a second TRA is disclosed. This second TRA is presented by HLA-Cw16 molecules, also known as HLA-C*1601. The disclosure shows that a given TRAP can yield a plurality of TRAs.

In U.S. Patent 5,487,974, incorporated by reference herein, tyrosinase is described as a tumor rejection antigen precursor. This reference discloses that a molecule which is produced by some normal cells (e.g., melanocytes), is processed in tumor cells to yield a tumor rejection antigen that is presented by HLA-A2 molecules.

In U.S. Patent No. 5,620,886, incorporated herein by reference in its entirety, a second TRA, not derived from tyrosinase is taught to be presented by HLA-A2 molecules. The TRA is derived from a TRAP, but is coded for by a known MAGE gene. This disclosure shows that a particular HLA molecule may present TRAs derived from different sources.

Additional TRAPs are disclosed in U.S. Patent Nos. 5,571,711, 5,610,013, 5,587,289 and 5,589,334, as well as PCT publication WO96/10577. The TRAPs are processed to tumor rejection antigens, which are presented by a variety of HLA molecules.

Summary of the Invention

It now has been discovered that the human macrophage colony stimulating factor gene (M-CSF, also known as colony stimulating factor-1 (CSF-1)) encodes a tumor rejection antigen precursor as an alternative open reading frame. The alternative open reading frame of M-CSF (alt.M-CSF ORF) is translated in a renal cell carcinoma cells. This open reading frame can be referred to as tumor-associated open reading frame (TORF) which contains a tumor associated antigen. Peptides derived from the alt.M-CSF ORF, when presented by an antigen presenting cell having an HLA class I molecule, effectively induce the activation and proliferation of $CD8^+$ cytotoxic T lymphocytes.

According to one aspect of the invention, an isolated polypeptide comprising the amino acid sequence of SEQ ID NO:5, or a fragment thereof is provided. Also provides is an isolated immunogenic polypeptide comprising the amino acid sequence of SEQ ID NO:12, or

a functional variant thereof. In some embodiments, the isolated immunogenic polypeptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:9 and SEQ ID NO:12. In a preferred embodiment, the isolated polypeptide consists of the amino acid sequence of SEQ ID NO:12. In other embodiments, the foregoing isolated polypeptides are non-hydrolyzable. Preferred non-hydrolyzable polypeptides are selected from the group consisting of peptides comprising D-amino acids, peptides comprising a -psi[CH_2NH]-reduced amide peptide bond, peptides comprising a -psi[$COCH_2$]-ketomethylene peptide bond, peptides comprising a -psi[CH(CN)NH]-(cyanomethylene) amino peptide bond, peptides comprising a -psi[$CH_2CH(OH)$]-hydroxyethylene peptide bond, peptides comprising a -psi[CH_2O]-peptide bond, and peptides comprising a -psi[CH_2S]-thiomethylene peptide bond.

According to another aspect of the invention, compositions comprising the foregoing isolated polypeptides, fragments and functional variants thereof are provided. In certain embodiments, the compositions include an isolated non-alt.M-CSF tumor rejection antigen peptide or a precursor thereof. In some embodiments the compositions include an isolated immunogenic polypeptide which includes the amino acid sequence of SEQ ID NO:12, preferably an amino acid sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:9, and SEQ ID NO:12.

According to still another aspect of the invention, isolated nucleic acids are provided. The isolated nucleic acids encode the foregoing polypeptides and immunogenic polypeptides. Preferably, the isolated nucleic acid, when translated, does not encode M-CSF, a precursor of M-CSF, or a fragment M-CSF. In certain embodiments, the isolated nucleic acid includes the nucleotide sequence set forth as SEQ ID NO:11.

According to still another aspect of the invention, expression vectors are provided which include the foregoing nucleic acids operably linked to a promoter. In some embodiments, the expression vectors include a nucleic acid which encodes an HLA class I molecule, preferably an HLA-B*3501 molecule. Host cells transfected or transformed with the foregoing expression vectors also are provided. In some embodiments, the host cells express an HLA class I molecule, preferably an HLA-B*3501 molecule.

Methods for enriching selectively a population of T lymphocytes with $CD8^+$ T lymphocytes specific for an alt.M-CSF immunogenic polypeptide are provided according to another aspect of the invention. The methods include contacting an isolated population of T

lymphocytes with an agent presenting a complex of the alt.M-CSF immunogenic polypeptide and an HLA class I molecule in an amount sufficient to selectively enrich the isolated population of T lymphocytes with the $CD8^+$ T lymphocytes. In some embodiments, the agent is an antigen presenting cell contacted with an alt.M-CSF immunogenic polypeptide comprising the amino acid sequence of SEQ ID NO:12. In other embodiments, the HLA class I molecule is an HLA-B*3501 molecule and the alt.M-CSF immunogenic polypeptide is selected from the group consisting of a peptide consisting of the amino acid sequence of SEQ ID NO:5, a peptide consisting of the amino acid sequence of SEQ ID NO:9, and a peptide consisting of the amino acid sequence of SEQ ID NO:12.

Methods for diagnosing a disorder characterized by expression of an alt.M-CSF polypeptide also are provided according to yet another aspect of the invention. The methods include contacting a biological sample isolated from a subject with an agent that is specific for the alt.M-CSF polypeptide, and determining the interaction between the agent and the alt.M-CSF polypeptide as a determination of the disorder. In some embodiments the alt.M-CSF polypeptide includes the amino acid sequence of SEQ ID NO:12; preferably the alt.M-CSF polypeptide consists of the amino acid sequence of SEQ ID NO:5 or SEQ ID NO:12.

According to still another aspect of the invention, methods for diagnosing a disorder characterized by expression of an alt.M-CSF immunogenic polypeptide which forms a complex with an HLA class I molecule are provided. The methods include contacting a biological sample isolated from a subject with an agent that binds the complex and determining binding between the complex and the agent as a determination of the disorder. In some embodiments, the HLA class I molecule is an HLA-B*3501 molecule and the alt.M-CSF immunogenic polypeptide includes the amino acid sequence of SEQ ID NO:12. In other embodiments the alt.M-CSF polypeptide consists of the amino acid sequence of SEQ ID NO:5 or SEQ ID NO:12.

According to yet another aspect of the invention, methods for treating a subject having a disorder characterized by expression of alt.M-CSF are provided. The methods include administering to the subject an amount of an alt.M-CSF immunogenic polypeptide sufficient to ameliorate the disorder. In some embodiments, the alt.M-CSF immunogenic polypeptide includes the amino acid sequence of SEQ ID NO:12, or a functional variant thereof. In other embodiments the alt.M-CSF polypeptide includes the amino acid sequence of SEQ ID NO:12; preferably the alt.M-CSF immunogenic polypeptide consists of the amino acid sequence of

SEQ ID NO:5, SEQ ID NO:9 or SEQ ID NO:12.

Methods for treating a subject having a disorder characterized by expression of alt.M-CSF are provided in another aspect of the invention. The methods include administering to the subject an amount of an agent which enriches selectively in the subject the presence of complexes of an HLA class I molecule and an alt.M-CSF immunogenic polypeptide, sufficient to ameliorate the disorder. In some embodiments, the HLA class I molecule is an HLA-B*3501 molecule and the alt.M-CSF immunogenic polypeptide includes the amino acid sequence of SEQ ID NO:12, or a functional variant thereof. Preferably the alt.M-CSF polypeptide consists of the amino acid sequence of SEQ ID NO:5, SEQ ID NO:9 or SEQ ID NO:12.

According to another aspect of the invention, methods for treating a subject having a disorder characterized by expression of alt.M-CSF are provided. The methods include administering to the subject an amount of autologous $CD8^+$ T lymphocytes sufficient to ameliorate the disorder. The $CD8^+$ T lymphocytes are specific for complexes of an HLA class I molecule and an alt.M-CSF immunogenic polypeptide. In some embodiments, the HLA class I molecule is an HLA-B*3501 molecule and the alt.M-CSF immunogenic polypeptide includes the amino acid sequence of SEQ ID NO:12, or a functional variant thereof. Preferably the alt.M-CSF polypeptide consists of the amino acid sequence of SEQ ID NO:5, SEQ ID NO:9 or SEQ ID NO:12.

According to still another aspect of the invention, methods for identifying functional variants of an alt.M-CSF immunogenic polypeptide are provided. The methods include selecting an alt.M-CSF immunogenic polypeptide, an HLA class I binding molecule which binds the alt.M-CSF immunogenic polypeptide or fragment thereof, and a T lymphocyte which is stimulated by the alt.M-CSF immunogenic polypeptide or fragment thereof presented by the HLA class I binding molecule. A first amino acid residue of the alt.M-CSF immunogenic polypeptide is added, deleted or substituted to prepare a variant peptide. The binding of the variant peptide to HLA class I binding molecule and/or the stimulation of the T lymphocyte is determined, wherein binding of the variant peptide to the HLA class I binding molecule and/or stimulation of the T lymphocyte by the variant peptide presented by the HLA class I binding molecule indicates that the variant peptide is a functional variant of the alt.M-CSF immunogenic polypeptide. In certain embodiments, the alt.M-CSF immunogenic polypeptide includes the amino acid sequence of SEQ ID NO:12. In other embodiments, the

methods include comparing the stimulation of the T lymphocyte by the alt.M-CSF immunogenic polypeptide and the stimulation of the T lymphocyte by the functional variant as a determination of the effectiveness of the stimulation of the T lymphocyte by the functional variant. In still other embodiments, the methods also include a step of adding, deleting or substituting at least one second amino acid to prepare a variant peptide, and determining the binding of the variant peptide to HLA class I binding molecule and/or the stimulation of the T lymphocyte. Binding of the variant peptide to the HLA class I binding molecule and/or stimulation of the T lymphocyte by the variant peptide presented by the HLA class I binding molecule indicates that the variant peptide having a second added, deleted or substituted amino acid is a functional variant of the alt.M-CSF immunogenic polypeptide.

Isolated polypeptides are provided according to another aspect of the invention. The isolated polypeptides bind selectively any of the foregoing alt.M-CSF polypeptides or immunogenic polypeptides, or fragments or variants thereof, provided that the isolated polypeptide is not an HLA class I molecule. In some embodiments, the isolated polypeptide is an antibody, preferably a monoclonal antibody. More preferably, the antibody is a chimeric antibody or a humanized antibody. In other embodiments, the isolated polypeptide is an antibody fragment selected from the group consisting of a Fab fragment, a $F(ab)_2$ fragment or a fragment including a CDR3 region selective for an alt.M-CSF polypeptide.

According to another aspect of the invention, isolated $CD8^+$ T lymphocytes which selectively bind a complex of an HLA class I molecule and an alt.M-CSF immunogenic polypeptide are provided. In some embodiments, the HLA class I molecule is an HLA-B*3501 molecule and the alt.M-CSF immunogenic polypeptide includes the amino acid sequence of SEQ ID NO:12, or a functional variant thereof. Preferably the alt.M-CSF polypeptide consists of the amino acid sequence of SEQ ID NO:5, SEQ ID NO:9 or SEQ ID NO:12.

Also provided according another aspect of the invention are isolated antigen presenting cells which include a complex of an HLA class I molecule and an alt.M-CSF immunogenic polypeptide. In some embodiments, the HLA class I molecule is an HLA-B*3501 molecule and the alt.M-CSF immunogenic polypeptide includes the amino acid sequence of SEQ ID NO:12, or a functional variant thereof. Preferably the alt.M-CSF polypeptide consists of the amino acid sequence of SEQ ID NO:5, SEQ ID NO:9 or SEQ ID NO:12.

According to yet another aspect of the invention, methods for identifying a candidate mimetic of an alt.M-CSF immunogenic polypeptide are provided. The methods include providing a HLA molecule which binds an alt.M-CSF immunogenic polypeptide or a fragment thereof, contacting the HLA molecule with a test molecule, and determining the binding of the test molecule to the HLA molecule. A test molecule which binds to the HLA molecule is a candidate mimetic of an alt.M-CSF immunogenic polypeptide. In some embodiments, the methods also include forming a complex of the HLA molecule and the candidate mimetic, contacting the complex with a T cell which binds to a complex of an HLA molecule and an alt.M-CSF immunogenic polypeptide, and assaying activation of the T cell. Preferably, activation of the T cell is indicated by a property selected from the group consisting of proliferation of the T cell, interferon-γ production by the T cell, tumor necrosis factor production by the T cell, and cytolysis of a target cell by the T cell.

According to still another aspect of the invention, vaccine compositions are provided. The vaccine compositions include a nucleic acid encoding an alt.M-CSF immunogenic polypeptide including the amino acid sequence of SEQ ID NO:5 or an immunogenic fragment thereof. In some embodiments, the nucleic acid is contained in a vector selected from the group consisting of adenoviruses, adeno-associated viruses, poxviruses, vaccinia viruses, attenuated poxviruses, Semliki Forest virus, Venezuelan equine encephalitis virus, retroviruses, Sindbis virus, Ty virus-like particle and recombinant bacteria. In other embodiments, the alt.M-CSF immunogenic polypeptide or immunogenic fragment thereof includes the amino acid sequence of SEQ ID NO:12. In certain embodiments, the vaccine compositions further includes a nucleic acid encoding a non-alt.M-CSF immunogenic polypeptide or an immunogenic fragment thereof.

Also provided according to another aspect of the invention are vaccine compositions including an immunogenic fragment of SEQ ID NO:5; preferably the immunogenic fragment includes the amino acid sequence of SEQ ID NO:12. In some embodiments the vaccine composition also includes a non-alt.M-CSF immunogenic polypeptide or an immunogenic fragment thereof.

According to still another aspect of the invention, vaccine compositions are provides which include a cell which expresses an alt.M-CSF nucleic acid or polypeptide, or an immunogenic fragment thereof. In certain embodiments, the vaccine compositions also include a non-alt.M-CSF nucleic acid or polypeptide, or an immunogenic fragment thereof.

In certain embodiments, the foregoing vaccine compositions include an adjuvant and/or a pharmaceutically acceptable carrier.

The invention also provides pharmaceutical preparations containing any one or more of the compositions described herein. Such pharmaceutical preparations can include pharmaceutically acceptable diluent carriers or excipients. The use of such compositions in the preparation of medicaments, particularly medicaments for the treatment of cancer also is provided.

In certain embodiments of the foregoing products, compositions and methods, additional preferred HLA class I molecules include HLA-A24 and HLA-Cw4. In still other embodiments, the agents, CTLs, and/or HLA molecules can be targeted to tumor tissue.

These and other objects of the invention will be described in further detail in connection with the detailed description of the invention.

Brief Description of the Figures

Fig. 1 shows the specificity of lysis by CTL clone 403A/9 for renal cell carcinoma cells.

Fig. 2 depicts the HLA-class I restriction of CTL 403A/9, and isolation of the cDNA pool D77 encoding the HLA-B35-restricted antigen recognized by CTL clone 403A/9.

Fig. 3 depicts the isolation of two cDNAs (pD77-314 and pD77-344) from pool D77 which both encode the human M-CSF.

Fig. 4 shows the alignment of a portion of the major (M-CSF; SEQ ID NOs:50 and 51) and the entire alternative (alt.M-CSF; SEQ ID NOs:4 and 5) open reading frames of M-CSF. The sequence of the peptide encoded by minigene MPO 29/39 is indicated (SEQ ID NO:9). The immunogenic peptide LPAVVGLSPGEQEY is boxed (SEQ ID NO:12). Position +1 in the major M-CSF protein sequence indicates the first residue of the mature M-CSF. Amino acids to the left of position +1 correspond to the leader sequence.

Fig. 5 demonstrates that the alt.M-CSF open reading frame encodes the peptide recognized by CTL 403A/9.

Fig. 6 depicts lysis by CTL 403A/9 of HLA-B*3501 positive EBV-transformed B-cells (HA7-EBV) incubated with the indicated peptides.

Fig. 7 demonstrates that the fourteen-mer peptide LPAVVGLSPGEQEY (SEQ ID NO:12) is the optimal peptide recognized by CTL 403A/9.

Fig. 8 shows the recognition of short term PTEC cell lines by CTL 403A/9.

Fig. 9 shows the correlation of M-CSF secretion and alt.M-CSF expression in several cancer cells.

Detailed Description of the Invention

The invention provides isolated alt.M-CSF peptides, some of which are presented by HLA class I molecules, which peptides stimulate the proliferation and activation of $CD8^+$ T lymphocytes. Such peptides are referred to herein as "alt.M-CSF immunogenic polypeptides". Hence, one aspect of the invention is an isolated peptide which includes the amino acid sequence of SEQ ID NO:12.

The examples below show the isolation of peptides which are alt.M-CSF immunogenic polypeptides. These exemplary peptides are processed translation products of the nucleic acid of SEQ ID NO:4. As such, it will be appreciated by one of ordinary skill in the art that the translation products from which an alt.M-CSF immunogenic polypeptide is processed to a final form for presentation may be of any length or sequence so long as they encompass the minimum alt.M-CSF immunogenic polypeptide, SEQ ID NO:12. As demonstrated in the examples below, peptides or proteins as small as 14 amino acids and as large as the amino acid sequence of the alt.M-CSF protein (SEQ ID NO:5) are appropriately processed, presented by HLA class I molecules and effective in stimulating $CD8^+$ T lymphocytes. The peptide of SEQ ID NO:12 may have one, two, three, four, five, six, seven, eight, nine, ten, or more amino acids added to either or both ends. The added amino acids can correspond to the alt.M-CSF polypeptide (SEQ ID NO:5), or can be unrelated. The antigenic portion of such a peptide is cleaved out under physiological conditions for presentation by HLA class I molecules.

Additional immunogenic peptides derived from the alt.M-CSF polypeptide may provoke an immune response when presented by HLA class I or class II molecules. The invention embraces all such immunogenic fragments of the alt.M-CSF polypeptide.

As used herein with respect to polypeptides, "isolated" means separated from its native environment and present in sufficient quantity to permit its identification or use. Isolated, when referring to a protein or polypeptide, means, for example: (i) selectively produced by expression cloning or (ii) purified as by chromatography or electrophoresis. Isolated proteins or polypeptides may be, but need not be, substantially pure. The term

"substantially pure" means that the proteins or polypeptides are essentially free of other substances with which they may be found in nature or *in vivo* systems to an extent practical and appropriate for their intended use. Substantially pure polypeptides may be produced by techniques well known in the art. Because an isolated protein may be admixed with a pharmaceutically acceptable carrier in a pharmaceutical preparation, the protein may comprise only a small percentage by weight of the preparation. The protein is nonetheless isolated in that it has been separated from the substances with which it may be associated in living systems, i.e. isolated from other proteins.

As noted above, the invention embraces functional variants of the alt.M-CSF immunogenic polypeptide. As used herein, a "functional variant" or "variant" of an alt.M-CSF immunogenic polypeptide is a molecule which contains one or more modifications to the primary amino acid sequence of an alt.M-CSF immunogenic polypeptide and retains the HLA class I binding properties disclosed herein, as well as the ability to stimulate proliferation and/or activation of $CD8^+$ T lymphocytes. Modifications which create an alt.M-CSF immunogenic polypeptide functional variant can be made for example 1) to enhance a property of an alt.M-CSF immunogenic polypeptide, such as peptide stability in an expression system or the stability of protein-protein binding such as HLA-peptide binding; 2) to provide a novel activity or property to an alt.M-CSF immunogenic polypeptide, such as addition of an antigenic epitope or addition of a detectable moiety; or 3) to provide a different amino acid sequence that produces the same or similar T cell stimulatory properties. Modifications to an alt.M-CSF immunogenic polypeptide can be made to a nucleic acid which encodes the peptide, and can include deletions, point mutations, truncations, amino acid substitutions and additions of amino acids. Alternatively, modifications can be made directly to the polypeptide, such as by cleavage, addition of a linker molecule, addition of a detectable moiety, such as biotin, addition of a fatty acid, substitution of one amino acid for another and the like. Modifications also embrace fusion proteins comprising all or part of the alt.M-CSF immunogenic polypeptide amino acid sequence.

The amino acid sequence of alt.M-CSF immunogenic polypeptides may be of natural or non-natural origin, that is, they may comprise a natural alt.M-CSF immunogenic polypeptide molecule or may comprise a modified sequence as long as the amino acid sequence retains the ability to stimulate cytolytic T cells when presented and retains the property of binding to an HLA class I molecule such as an HLA B*3501 molecule. For

example, alt.M-CSF immunogenic polypeptides in this context may be fusion proteins of an alt.M-CSF immunogenic polypeptide and unrelated amino acid sequences, synthetic peptides of amino acid sequences shown in SEQ ID NOs:5, 9, and 12, labeled peptides, peptides isolated from patients with an alt.M-CSF expressing cancer, peptides isolated from cultured cells which express alt.M-CSF, peptides coupled to nonpeptide molecules (for example in certain drug delivery systems) and other molecules which include the amino acid sequence of SEQ ID NO:12.

Preferably, alt.M-CSF immunogenic polypeptides are non-hydrolyzable. To provide such peptides, one may select alt.M-CSF immunogenic polypeptides from a library of non-hydrolyzable peptides, such as peptides containing one or more D-amino acids or peptides containing one or more non-hydrolyzable peptide bonds linking amino acids. Alternatively, one can select peptides which are optimal for inducing $CD8^+$ T lymphocytes and then modify such peptides as necessary to reduce the potential for hydrolysis by proteases. For example, to determine the susceptibility to proteolytic cleavage, peptides may be labeled and incubated with cell extracts or purified proteases and then isolated to determine which peptide bonds are susceptible to proteolysis, e.g., by sequencing peptides and proteolytic fragments. Alternatively, potentially susceptible peptide bonds can be identified by comparing the amino acid sequence of an alt.M-CSF immunogenic polypeptide with the known cleavage site specificity of a panel of proteases. Based on the results of such assays, individual peptide bonds which are susceptible to proteolysis can be replaced with non-hydrolyzable peptide bonds by *in vitro* synthesis of the peptide.

Many non-hydrolyzable peptide bonds are known in the art, along with procedures for synthesis of peptides containing such bonds. Non-hydrolyzable bonds include -psi[CH_2NH]- reduced amide peptide bonds, -psi[$COCH_2$]- ketomethylene peptide bonds, -psi[CH(CN)NH]- (cyanomethylene)amino peptide bonds, -psi[$CH_2CH(OH)$]- hydroxyethylene peptide bonds, -psi[CH_2O]- peptide bonds, and -psi[CH_2S]- thiomethylene peptide bonds.

Nonpeptide analogs of peptides, e.g., those which provide a stabilized structure or lessened biodegradation, are also contemplated. Peptide mimetic analogs can be prepared based on a selected alt.M-CSF immunogenic polypeptide by replacement of one or more residues by nonpeptide moieties. Preferably, the nonpeptide moieties permit the peptide to retain its natural confirmation, or stabilize a preferred, e.g., bioactive, confirmation. One example of methods for preparation of nonpeptide mimetic analogs from peptides is described

in Nachman et al., *Regul. Pept.* 57:359-370 (1995). Peptide mimetics also can be selected from libraries of synthetic compounds (e.g. combinatorial libraries of small organic molecules) or natural molecules according to the HLA binding properties and/or T cell stimulatory properties of such molecule. Assays for identification of mimetics of an alt.M-CSF immunogenic polypeptide from libraries such as binding assays are well known in the art. Peptide as used herein embraces all of the foregoing.

If a variant involves a change to an amino acid of SEQ ID NO:12, functional variants of the alt.M-CSF immunogenic polypeptide having conservative amino acid substitutions typically will be preferred, i.e., substitutions which retain a property of the original amino acid such as charge, hydrophobicity, conformation, etc. Examples of conservative substitutions of amino acids include substitutions made amongst amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D.

Other methods for identifying functional variants of the alt.M-CSF immunogenic polypeptides are provided in a published PCT application of Strominger and Wucherpfennig (US/96/03182). These methods rely upon the development of amino acid sequence motifs to which potential epitopes may be compared. Each motif describes a finite set of amino acid sequences in which the residues at each (relative) position may be (a) restricted to a single residue, (b) allowed to vary amongst a restricted set of residues, or (c) allowed to vary amongst all possible residues. For example, a motif might specify that the residue at a first position may be any one of the residues valine, leucine, isoleucine, methionine, or phenylalanine; that the residue at the second position must be histidine; that the residue at the third position may be any amino acid residue; that the residue at the fourth position may be any one of the residues valine, leucine, isoleucine, methionine, phenylalanine, tyrosine or tryptophan; and that the residue at the fifth position must be lysine.

Sequence motifs for alt.M-CSF immunogenic polypeptide functional variants can be developed by analysis of the binding domains or binding pockets of major histocompatibility complex HLA class I proteins (e.g., HLA-A24, HLA-B*3501, HLA-Cw4) and/or the T cell receptor ("TCR") contact points of the alt.M-CSF immunogenic polypeptides disclosed herein. By providing a detailed structural analysis of the residues involved in forming the HLA class I binding pockets, one of ordinary skill in the art is enabled to make predictions of sequence motifs for binding to any of the HLA class I proteins.

Using these sequence motifs as search, evaluation, or design criteria, one of ordinary

skill in the art is enabled to identify classes of peptides (functional variants of the alt.M-CSF immunogenic polypeptides disclosed herein) which have a reasonable likelihood of binding to a particular HLA molecule and of interacting with a T cell receptor to induce T cell response. These peptides can be synthesized and tested for activity as described herein. Use of these motifs, as opposed to pure sequence homology (which excludes many peptides which are antigenically similar but quite distinct in sequence) or sequence homology with unlimited "conservative" substitutions (which admits many peptides which differ at critical highly conserved sites), represents a method by which one of ordinary skill in the art can evaluate peptides for potential application in the treatment of disease.

The Strominger and Wucherpfennig PCT application, and references cited therein, all of which are incorporated by reference, describe the HLA class II and TCR binding pockets which contact residues of an HLA class II peptide. Likewise, by keeping the residues which are likely to bind in the HLA class I and/or TCR binding pockets constant or permitting only specified substitutions, functional variants of the alt.M-CSF immunogenic polypeptides can be prepared which retain binding to HLA class I and T cell receptor.

Localization of one or more antigenic peptides in a protein sequence can be aided by HLA peptide binding predictions made according to established rules for binding potential (e.g., Parker et al, *J. Immunol.* 152:163, 1994; Rammensee et al., *Immunogenetics* 41:178-228, 1995). HLA binding predictions can conveniently be made using an algorithm available via the Internet on the National Institutes of Health World Wide Web site at URL http://bimas.dcrt.nih.gov.

Thus methods for identifying functional variants of an alt.M-CSF immunogenic polypeptide are provided. In general, the methods include selecting an alt.M-CSF immunogenic polypeptide, an HLA class I binding molecule which binds the alt.M-CSF immunogenic polypeptide, and a T cell which is stimulated by the alt.M-CSF immunogenic polypeptide presented by the HLA class I binding molecule. In preferred embodiments, the alt.M-CSF immunogenic polypeptide comprises the amino acid sequence of SEQ ID NO:12. More preferably, the peptide consists of the amino acid sequence of SEQ ID NO:5,SEQ ID NO:9, or SEQ ID NO:12. A first amino acid residue of the alt.M-CSF immunogenic polypeptide is mutated to prepare a variant peptide. The amino acid residue can be mutated according to the principles of HLA and T cell receptor contact points set forth above. Any method for preparing variant peptides can be employed, such as synthesis of the variant

peptide, recombinantly producing the variant peptide using a mutated nucleic acid molecule, and the like.

The binding of the variant peptide to HLA class I binding molecule and/or stimulation of the T cell are then determined according to standard procedures. For example, as exemplified below, the variant peptide can be contacted with an antigen presenting cell which contains the HLA class I molecule which binds the alt.M-CSF immunogenic polypeptide to form a complex of the variant peptide and antigen presenting cell. This complex can then be contacted with a T cell which recognizes the alt.M-CSF immunogenic polypeptide presented by the HLA class I binding molecule. T cells can be obtained from a patient having a condition characterized by expression of alt.M-CSF. Recognition of variant peptides by the T cells can be determined by measuring an indicator of T cell stimulation such as TNF or IFNγ production.

Binding of the variant peptide to the HLA class I binding molecule and/or stimulation of the T cell by the variant peptide presented by the HLA class I binding molecule indicates that the variant peptide is a functional variant. The methods also can include the step of comparing the stimulation of the T cell by the alt.M-CSF immunogenic polypeptide and the stimulation of the T cell by the functional variant as a determination of the effectiveness of the stimulation of the T cell by the functional variant. By comparing the functional variant with the alt.M-CSF immunogenic polypeptide, peptides with increased T cell stimulatory properties can be prepared. Iterative application of the method described above can be used to prepare variant peptides with 2 or more amino acid differences from the alt.M-CSF immunogenic peptides.

Variants of the alt.M-CSF immunogenic polypeptides prepared by any of the foregoing methods can be sequenced, if necessary, to determine the amino acid sequence and thus deduce the nucleotide sequence which encodes such variants.

Thus those nucleic acid sequences which code for an alt.M-CSF immunogenic polypeptide or variant thereof, including allelic variants, are also a part of the invention. In screening for nucleic acids which encode an alt.M-CSF immunogenic polypeptide, a nucleic acid hybridization such as a Southern blot or a Northern blot may be performed under stringent conditions, together with a ^{32}P probe. The term "stringent conditions" as used herein refers to parameters with which the art is familiar. Nucleic acid hybridization parameters may be found in references which compile such methods, e.g. *Molecular Cloning: A Laboratory*

Manual, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. Exemplary stringent conditions include hybridization at 65°C in hybridization buffer (3.5 x SSC, 0.02% Ficoll, 0.02% Polyvinyl pyrolidone, 0.02% Bovine Serum Albumin, 25mM NaH_2PO_4 (pH7), 0.5% SDS, 2mM EDTA). SSC is 0.15M Sodium Chloride/0.015M Sodium Citrate, pH 7; SDS is Sodium Dodecyl Sulphate; and EDTA is Ethylene diaminetetraacetic acid. After hybridization, the membrane upon which the DNA is transferred can be washed, for example, in 2x SSC at room temperature and then in 0.1x SSC/0.1xSDS at temperatures up to 68°C. After washing the membrane to which DNA encoding an alt.M-CSF immunogenic polypeptide was finally transferred, the membrane can be placed against X-ray film to detect the radioactive signal.

There are other conditions, reagents, and so forth which can used, which result in a similar degree of stringency. The skilled artisan will be familiar with such conditions, and thus they are not given here. It will be understood, however, that the skilled artisan will be able to manipulate the conditions in a manner to permit the clear identification of homologs and alleles of nucleic acids encoding the alt.M-CSF immunogenic polypeptides of the invention. The skilled artisan also is familiar with the methodology for screening cells and libraries for expression of such molecules which then are routinely isolated, followed by isolation of the pertinent nucleic acid molecule and sequencing.

As used herein with respect to nucleic acids, the term "isolated" means: (i) amplified *in vitro* by, for example, polymerase chain reaction (PCR); (ii) recombinantly produced by cloning; (iii) purified, as by cleavage and gel separation; or (iv) synthesized by, for example, chemical synthesis. An isolated nucleic acid is one which is readily manipulable by recombinant DNA techniques well known in the art. Thus, a nucleotide sequence contained in a vector in which 5' and 3' restriction sites are known or for which polymerase chain reaction (PCR) primer sequences have been disclosed is considered isolated but a nucleic acid sequence existing in its native state in its natural host is not. An isolated nucleic acid may be substantially purified, but need not be. For example, a nucleic acid that is isolated within a cloning or expression vector is not pure in that it may comprise only a tiny percentage of the material in the cell in which it resides. Such a nucleic acid is isolated, however, as the term is used herein because it is readily manipulable by standard techniques known to those of ordinary skill in the art. An isolated nucleic acid as used

herein is not a naturally occurring chromosome.

The invention also includes the use of nucleic acid sequences which include alternative codons that encode the same amino acid residues of the alt.M-CSF immunogenic polypeptides. For example, as disclosed herein, the peptide LPAVVGLSPGEQEY (SEQ ID NO:12) is an alt.M-CSF immunogenic polypeptide. The leucine residues can be encoded by the codons CUA, CUC, CUG, CUU, UUA and UUG. Each of the six codons is equivalent for the purposes of encoding a leucine residue. Thus, it will be apparent to one of ordinary skill in the art that any of the leucine-encoding nucleotide triplets may be employed to direct the protein synthesis apparatus, *in vitro* or *in vivo*, to incorporate a leucine residue. Similarly, nucleotide sequence triplets which encode other amino acid residues comprising the alt.M-CSF immunogenic polypeptide of SEQ ID NO:12 include: GUA, GUC, GUG and GUU (valine codons); GCU, GCC, GCG, GCA (alanine codons); CCU, CCC, CCA, CCG (proline codons); GGU, GGA, GGG, GGC (glycine codons); GAA and GAG (glutamine codons); GAA and GAG (glutamic acid codons); UAC and UAU (tyrosine codons). Other amino acid residues may be encoded similarly by multiple nucleotide sequences. Thus, the invention embraces degenerate nucleic acids that differ from the native alt.M-CSF immunogenic polypeptide encoding nucleic acids in codon sequence due to the degeneracy of the genetic code.

The invention also provides modified nucleic acid molecules which include additions, substitutions and deletions of one or more nucleotides. In preferred embodiments, these modified nucleic acid molecules and/or the polypeptides they encode retain at least one activity or function of the unmodified nucleic acid molecule and/or the polypeptides, such as antigenicity, enzymatic activity, receptor binding, formation of complexes by binding of peptides by MHC class I or class II molecules, etc. In certain embodiments, the modified nucleic acid molecules encode modified polypeptides, preferably polypeptides having conservative amino acid substitutions as are described elsewhere herein. The modified nucleic acid molecules are structurally related to the unmodified nucleic acid molecules and in preferred embodiments are sufficiently structurally related to the unmodified nucleic acid molecules so that the modified and unmodified nucleic acid molecules hybridize under stringent conditions known to one of skill in the art.

For example, modified nucleic acid molecules which encode polypeptides having single amino acid changes can be prepared. Each of these nucleic acid molecules can have

one, two or three nucleotide substitutions exclusive of nucleotide changes corresponding to the degeneracy of the genetic code as described herein. Likewise, modified nucleic acid molecules which encode polypeptides having two amino acid changes can be prepared which have, e.g., 2-6 nucleotide changes. Numerous modified nucleic acid molecules like these will be readily envisioned by one of skill in the art, including for example, substitutions of nucleotides in codons encoding amino acids 2 and 3, 2 and 4, 2 and 5, 2 and 6, and so on. In the foregoing example, each combination of two amino acids is included in the set of modified nucleic acid molecules, as well as all nucleotide substitutions which code for the amino acid substitutions. Additional nucleic acid molecules that encode polypeptides having additional substitutions (i.e., 3 or more), additions or deletions (e.g., by introduction of a stop codon or a splice site(s)) also can be prepared and are embraced by the invention as readily envisioned by one of ordinary skill in the art. Any of the foregoing nucleic acids or polypeptides can be tested by routine experimentation for retention of structural relation or activity to the nucleic acids and/or polypeptides disclosed herein.

It will be understood that while the nucleotide sequence of the alt.M-CSF polypeptide is identical to M-CSF encoding nucleotide sequence (albeit shorter), the reading frame of the alt.M-CSF polypeptide is different than that which encodes the macrophage colony stimulating factor polypeptide. Preferred nucleic acids encoding alt.M-CSF polypeptides are those which preferentially express alt.M-CSF immunogenic polypeptides. More preferably, the nucleic acids are incapable of encoding M-CSF. Thus preferred nucleic acids encoding alt.M-CSF polypeptides will be constructed so that only the alt.M-CSF polypeptide reading frame is translated. Alternatively, the nucleotide sequence of the nucleic acid can be altered such that alt.M-CSF polypeptide, but not the wild-type M-CSF, is expressed. For example, altering the fourth nucleotide of SEQ ID NO:4 from guanine to adenosine (G-->A) alters the codon in the M-CSF reading frame from a tryptophan codon to a stop codon (TGG-->TGA). This change also would alter the alt.M-CSF second amino acid from alanine to threonine (GCT-->ACT codon change). Such a change would not affect the sequence of the alt.M-CSF immunogenic polypeptide (SEQ ID NO:12), but would stop translation of the M-CSF polypeptide. Other similar changes could be made to the nucleotide sequence to affect the translation.

It will also be understood that the invention embraces the use of the sequences in expression vectors including recombinant plasmids, phagemids, viruses and the like, as well

as to transfect host cells and cell lines, be these prokaryotic (e.g., *E. coli*), or eukaryotic (e.g., dendritic cells, CHO cells, COS cells, yeast expression systems and recombinant baculovirus expression in insect cells). The expression vectors require that the pertinent sequence, i.e., those described *supra*, be operably linked to a promoter. Delivery of expression vectors containing the alt.M-CSF sequences *in vivo* and/or *in vitro* can be via the use of nucleic acid delivery systems known in the art (see, e.g., Allsopp et al., *Eur. J. Immunol.* 26(8):1951-1959, 1996). Recombinant vectors including viruses selected from the group consisting of adenoviruses, adeno-associated viruses, poxviruses including vaccinia viruses and attenuated poxviruses such as NYVAC, Semliki Forest virus, Venezuelan equine encephalitis virus, retroviruses, Sindbis virus, and Ty virus-like particle, plasmids (e.g. "naked" DNA), bacteria (e.g. the bacterium Bacille Calmette Guerin, BCG; attenuated *Salmonella*), and the like can be used in such delivery, for example, for use as a vaccine. Other viruses, expression vectors and the like which are useful in preparation of a vaccine are known to one of ordinary skill in the art. One can test the alt.M-CSF delivery systems in standard model sytems such as mice to determine efficacy of the delivery system. The systems also can be tested in human clinical trials.

Especially preferred are nucleic acids encoding a series of epitopes, known as "polytopes". The epitopes can be arranged in sequential or overlapping fashion (*see, e.g.*, Thomson et al., *Proc. Natl. Acad. Sci. USA* 92:5845-5849, 1995; Gilbert et al., *Nature Biotechnol.* 15:1280-1284, 1997), with or without the natural flanking sequences, and can be separated by unrelated linker sequences if desired. The polytope is processed to generated individual epitopes which are recognized by the immune system for generation of immune responses.

Thus, for example, alt.M-CSF immunogenic polypeptides such as SEQ ID NO:12, and which are presented by MHC molecules and recognized by CTLs (or T helper lymphocytes) can be combined with peptides from other tumor rejection antigens (e.g. by preparation of hybrid nucleic acids or polypeptides) to form "polytopes". Exemplary tumor associated peptide antigens that can be administered to induce or enhance an immune response are derived from tumor associated genes and encoded proteins including MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A7, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A11, MAGE-A12, MAGE-A13, GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7, GAGE-8, BAGE-1, RAGE-1, LB33/MUM-1, PRAME,

NAG, MAGE-Xp2 (MAGE-B2), MAGE-Xp3 (MAGE-B3), MAGE-Xp4 (MAGE-B4), tyrosinase, brain glycogen phosphorylase, Melan-A, MAGE-C1, MAGE-C2, NY-ESO-1, LAGE-1, SSX-1, SSX-2 (HOM-MEL-40), SSX-1, SSX-4, SSX-5, SCP-1 and CT-7. For example, antigenic peptides characteristic of tumors include those listed in Table I below.

Table I: Exemplary Antigens

Gene	MHC	Peptide	Position	SEQ ID NO:
MAGE-A1	HLA-A1	EADPTGHSY	161-169	16
	HLA-Cw16	SAYGEPRKL	230-238	17
MAGE-A3	HLA-A1	EVDPIGHLY	168-176	18
	HLA-A2	FLWGPRALV	271-279	19
	HLA-B44	MEVDPIGHLY	167-176	20
BAGE	HLA-Cw16	AARAVFLAL	2-10	21
GAGE-1,2	HLA-Cw16	YRPRPRRY	9-16	22
RAGE	HLA-B7	SPSSNRIRNT	11-20	23
GnT-V	HLA-A2	VLPDVFIRC(V)	2-10/11	24, 25
MUM-1	HLA-B44	EEKLIVVLF	exon 2/intron	26
		EEKLSVVLF (wild type)		27
CDK4	HLA-A2	ACDPHSGHFV	23-32	28
		ARDPHSGHFV (wild type)		29
β-catenin	HLA-A24	SYLDSGIHF	29-37	30
		SYLDSGIHS (wild type)		31
Tyrosinase	HLA-A2	MLLAVLYCL	1-9	32
	HLA-A2	YMNGTMSQV	369-377	33
	HLA-A2	YMDGTMSQV	369-377	49
	HLA-A24	AFLPWHRLF	206-214	34
	HLA-B44	SEIWRDIDF	192-200	35
	HLA-B44	YEIWRDIDF	192-200	36
	HLA-DR4	QNILLSNAPLGPQFP	56-70	37

	HLA-DR4	DYSYLQDSDPDSFQD	448-462	38
Melan-A^{MART-1}	HLA-A2	(E)AAGIGILTV	26/27-35	39, 40
	HLA-A2	ILTVILGVL	32-40	41
gp100^{Pmel117}	HLA-A2	KTWGQYWQV	154-162	42
	HLA-A2	ITDQVPFSV	209-217	43
	HLA-A2	YLEPGPVTA	280-288	44
	HLA-A2	LLDGTATLRL	457-466	45
	HLA-A2	VLYRYGSFSV	476-485	46
PRAME	HLA-A24	LYVDSLFFL	301-309	47
MAGE-6	HLA-Cw16	KISGGPRISYPL	292-303	48

Other examples will be known to one of ordinary skill in the art (for example, see Coulie, *Stem Cells* 13:393-403, 1995), and can be used in the invention in a like manner as those disclosed herein. One of ordinary skill in the art can prepare polypeptides comprising alt.M-CSF immunogenic polypeptides and one or more of the foregoing tumor rejection peptides, or nucleic acids encoding such polypeptides, according to standard procedures of molecular biology.

Thus polytopes are groups of two or more potentially immunogenic or immune response stimulating peptides which can be joined together in various arrangements (e.g. concatenated, overlapping). The polytope (or nucleic acid encoding the polytope) can be administered in a standard immunization protocol, e.g. to animals, to test the effectiveness of the polytope in stimulating, enhancing and/or provoking an immune response.

The peptides can be joined together directly or via the use of flanking sequences to form polytopes, and the use of polytopes as vaccines is well known in the art (see, e.g., Thomson et al., *Proc. Acad. Natl. Acad. Sci USA* 92(13):5845-5849, 1995; Gilbert et al., *Nature Biotechnol.* 15(12):1280-1284, 1997; Thomson et al., *J. Immunol.* 157(2):822-826, 1996; Tam et al., *J. Exp. Med.* 171(1):299-306, 1990). For example, Tam showed that polytopes consisting of both MHC class I and class II binding epitopes successfully generated antibody and protective immunity in a mouse model. Tam also demonstrated that polytopes comprising "strings" of epitopes are processed to yield individual epitopes which are presented by MHC molecules and recognized by CTLs. Thus polytopes containing various

numbers and combinations of epitopes can be prepared and tested for recognition by CTLs and for efficacy in increasing an immune response.

It is known that tumors express a set of tumor antigens, of which only certain subsets may be expressed in the tumor of any given patient. Polytopes can be prepared which correspond to the different combination of epitopes representing the subset of tumor rejection antigens expressed in a particular patient. Polytopes also can be prepared to reflect a broader spectrum of tumor rejection antigens known to be expressed by a tumor type. Polytopes can be introduced to a patient in need of such treatment as polypeptide structures, or via the use of nucleic acid delivery systems known in the art (see, e.g., Allsopp et al., *Eur. J. Immunol.* 26(8):1951-1959, 1996). Adenovirus, pox virus, Ty-virus like particles, adeno-associated virus, plasmids, bacteria, etc. can be used in such delivery. One can test the polytope delivery systems in mouse models to determine efficacy of the delivery system. The systems also can be tested in human clinical trials.

As it has been found that human HLA-B*3501 molecules present an alt.M-CSF immunogenic polypeptide, the expression vector may also include a nucleic acid sequence coding for an HLA-B*3501 molecule. Nucleic acids encoding single chain soluble HLA/peptide complex including an alt.M-CSF immunogenic polypeptide fused to an HLA-B*3501 molecule can be prepared as described by Lone et al. (*J. Immunother.* 21:283-294, 1998). Other HLA class I molecules which present alt.M-CSF peptides (e.g., HLA-A24, HLA-Cw4) can be used in a similar manner to HLA-B*3501 in all aspects of the invention.

In a situation where the vector contains both coding sequences, it can be used to transfect a cell which does not normally express either one. The alt.M-CSF immunogenic polypeptide coding sequence may be used alone, when, e.g. the host cell already expresses an HLA-B*3501 molecule. Of course, there is no limit on the particular host cell which can be used as the vectors which contain the two coding sequences may be used in host cells which do not express HLA-B*3501 molecules if desired, and the nucleic acid coding for the alt.M-CSF immunogenic polypeptide can be used in antigen presenting cells which express an HLA-B*3501 molecule.

In certain patients it may be necessary or preferred to target expression of a different HLA class I molecule to tumor cells for tumor-specific adoptive immunotherapy. For example, a patient expressing HLA-Cw4 but not HLA-B*3501 could be treated with a vector targeted to renal carcinoma cells (e.g., by RCC-specific monoclonal antibody, such as G250

or 138H11) for selective expression of HLA-B*3501 in RCC. Adoptive immunotherapy with CTLs that recognize alt.M-CSF presented by HLA-B*3501 then could be administered to promote selective cytolysis of renal carcinoma cells that express alt.M-CSF. Other therapeutics also could be targeted to renal carcinoma cells to increase selectivity, including alt.M-CSF and CTLs that recognize alt.M-CSF.

As used herein, a "vector" may be any of a number of nucleic acids into which a desired sequence may be inserted by restriction and ligation for transport between different genetic environments or for expression in a host cell. Vectors are typically composed of DNA although RNA vectors are also available. Vectors include, but are not limited to, plasmids, phagemids, bacteria and virus genomes as disclosed herein, such as adenovirus, poxvirus and BCG. A cloning vector is one which is able to replicate in a host cell or be replicated after its integration into the genome of a host cell, and which is further characterized by one or more endonuclease restriction sites at which the vector may be cut in a determinable fashion and into which a desired DNA sequence may be ligated such that the new recombinant vector retains its ability to replicate in the host cell. In the case of plasmids, replication of the desired sequence may occur many times as the plasmid increases in copy number within the host bacterium or just a single time per host before the host reproduces by mitosis. In the case of phage, replication may occur actively during a lytic phase or passively during a lysogenic phase. An expression vector is one into which a desired DNA sequence may be inserted by restriction and ligation such that it is operably joined to regulatory sequences and may be expressed as an RNA transcript. Vectors may further contain one or more marker sequences suitable for use in the identification of cells which have or have not been transformed or transfected with the vector. Markers include, for example, genes encoding proteins which increase or decrease either resistance or sensitivity to antibiotics or other compounds, genes which encode enzymes whose activities are detectable by standard assays known in the art (e.g., β-galactosidase, alkaline phosphatase or luciferase), and genes which visibly affect the phenotype of transformed or transfected cells, hosts, colonies or plaques (e.g., green fluorescent protein). Preferred vectors are those capable of autonomous replication and expression of the structural gene products present in the DNA segments to which they are operably joined.

As used herein, a coding sequence and regulatory sequences are said to be "operably" joined when they are covalently linked in such a way as to place the expression or

transcription of the coding sequence under the influence or control of the regulatory sequences. If it is desired that the coding sequences be translated into a functional protein, two DNA sequences are said to be operably joined if induction of a promoter in the 5' regulatory sequences results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a promoter region would be operably joined to a coding sequence if the promoter region were capable of effecting transcription of that DNA sequence such that the resulting transcript might be translated into the desired protein or polypeptide. As noted above, certain preferred nucleic acids express only alt.M-CSF polypeptides (and not M-CSF) by choice of reading frame.

The precise nature of the regulatory sequences needed for gene expression may vary between species or cell types, but shall in general include, as necessary, 5' non-transcribed and 5' non-translated sequences involved with the initiation of transcription and translation respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5' non-transcribed regulatory sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined gene. Regulatory sequences may also include enhancer sequences or upstream activator sequences as desired. The vectors of the invention may optionally include 5' leader or signal sequences. The choice and design of an appropriate vector is within the ability and discretion of one of ordinary skill in the art.

Expression vectors containing all the necessary elements for expression are commercially available and known to those skilled in the art. See, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, 1989. Cells are genetically engineered by the introduction into the cells of heterologous DNA (RNA) encoding an alt.M-CSF immunogenic polypeptide. That heterologous DNA (RNA) is placed under operable control of transcriptional elements to permit the expression of the heterologous DNA in the host cell.

Preferred systems for mRNA expression in mammalian cells are those such as pRc/CMV (available from Invitrogen, Carlsbad, CA) that contain a selectable marker such as a gene that confers G418 resistance (which facilitates the selection of stably transfected cell

lines) and the human cytomegalovirus (CMV) enhancer-promoter sequences. Additionally, suitable for expression in primate or canine cell lines is the pCEP4 vector (Invitrogen), which contains an Epstein Barr virus (EBV) origin of replication, facilitating the maintenance of plasmid as a multicopy extrachromosomal element. Another expression vector is the pEF-BOS plasmid containing the promoter of polypeptide Elongation Factor 1α, which stimulates efficiently transcription *in vitro*. The plasmid is described by Mishizuma and Nagata (*Nuc. Acids Res.* 18:5322, 1990), and its use in transfection experiments is disclosed by, for example, Demoulin (*Mol. Cell. Biol.* 16:4710-4716, 1996). Still another preferred expression vector is an adenovirus, described by Stratford-Perricaudet, which is defective for E1 and E3 proteins (*J. Clin. Invest.* 90:626-630, 1992). The use of the adenovirus to express proteins for immunization is disclosed by Warnier et al., in intradermal injection in mice for immunization against P1A (*Int. J. Cancer*, 67:303-310, 1996).

The invention also embraces so-called expression kits, which allow the artisan to prepare a desired expression vector or vectors. Such expression kits include at least separate portions of at least two of the previously discussed materials. Other components may be added, as desired.

The invention as described herein has a number of uses, some of which are described herein. First, the invention permits the artisan to diagnose a disorder characterized by expression of an alt.M-CSF immunogenic polypeptide. These methods involve determining expression of an alt.M-CSF immunogenic polypeptide, or a complex of an alt.M-CSF immunogenic polypeptide and an HLA class I molecule in a biological sample. The expression of a peptide or complex of peptide and HLA class I molecule can be determined by assaying with a binding partner for the peptide or complex, such as an antibody.

Preferably, the diagnostic methods involve contacting a biological sample isolated from a subject with an agent specific for the alt.M-CSF immunogenic polypeptide to detect the presence of the alt.M-CSF immunogenic polypeptide in the biological sample. As used herein, "contacting" means placing the biological sample in sufficient proximity to the agent and under the appropriate conditions of, e.g., concentration, temperature, time, ionic strength, to allow the specific interaction between the agent and alt.M-CSF immunogenic polypeptide that are present in the biological sample. In general, the conditions for contacting the agent with the biological sample are conditions known by those of ordinary skill in the art to facilitate a specific interaction between a molecule and its cognate (e.g., a protein and its

receptor cognate, an antibody and its protein antigen cognate, a nucleic acid and its complementary sequence cognate) in a biological sample. Exemplary conditions for facilitating a specific interaction between a molecule and its cognate are described in U.S. Patent No. 5,108,921, issued to Low et al.

The biological sample can be located *in vivo* or *in vitro*. For example, the biological sample can be a tissue *in vivo* and the agent specific for the alt.M-CSF immunogenic polypeptide can be used to detect the presence of such molecules in the tissue. Alternatively, the biological sample can be located *in vitro* (e.g., a blood sample, tumor biopsy, tissue extract). In a particularly preferred embodiment, the biological sample can be a cell-containing sample, more preferably a sample containing tumor cells.

The invention also permits the artisan to treat a subject having a disorder characterized by expression of an alt.M-CSF immunogenic polypeptide. Treatments include administering an agent which enriches in the subject a complex of an alt.M-CSF immunogenic polypeptide and an HLA class I molecule, and administering $CD8^+$ T lymphocytes which are specific for such complexes. Agents useful in the foregoing treatments include alt.M-CSF immunogenic polypeptides and functional variants thereof, complexes of such peptides and HLA class I binding molecules (e.g. HLA B*3501), antigen presenting cells bearing complexes of an alt.M-CSF immunogenic polypeptide and an HLA class I binding molecule, soluble single chain fusions of HLA and alt.M-CSF polypeptides, and the like. The invention also permits an artisan to selectively enrich a population of T lymphocytes for $CD8^+$ T lymphocytes specific for an alt.M-CSF immunogenic polypeptide.

The isolation of the alt.M-CSF immunogenic polypeptides also makes it possible to isolate or design nucleic acids which encode the alt.M-CSF immunogenic polypeptides. Nucleic acids can be used to produce *in vitro* or in prokaryotic or eukaryotic host cells the alt.M-CSF immunogenic polypeptides or proteins containing such polypeptides. A variety of methodologies well-known to the skilled practitioner can be utilized to obtain isolated alt.M-CSF immunogenic polypeptides. For example, an expression vector may be introduced into cells to cause production of the peptides. In another method, mRNA transcripts may be microinjected or otherwise introduced into cells to cause production of the encoded peptides. Translation of mRNA in cell-free extracts such as the reticulocyte lysate system also may be used to produce peptides. Peptides comprising the alt.M-CSF immunogenic polypeptide of the invention may also be synthesized *in vitro*. Those skilled in the art also can readily follow

known methods for isolating peptides in order to obtain isolated alt.M-CSF immunogenic polypeptides. These include, but are not limited to, immunochromotography, HPLC, size-exclusion chromatography, ion-exchange chromatography and immune-affinity chromatography.

These isolated alt.M-CSF immunogenic polypeptides, or complexes of the peptides and HLA class I molecules, such as an HLA-B*3501 molecule, may be combined with materials such as adjuvants to produce vaccines useful in treating disorders characterized by expression of the alt.M-CSF immunogenic polypeptide. In addition, vaccines can be prepared from cells which present the alt.M-CSF immunogenic polypeptide/HLA complexes on their surface, such as transfected dendritic cells, transfected B cells, non-proliferative transfectants, etcetera. In all cases where cells are used as a vaccine, these can be cells transfected with coding sequences for one or both of the components necessary to stimulate $CD8^+$ lymphocytes, or be cells which already express both molecules without the need for transfection. Vaccines also encompass expression vectors and naked DNA or RNA, encoding an alt.M-CSF immunogenic polypeptide, precursor thereof, or fusion protein thereof, which may be produced *in vitro* and administered via injection, particle bombardment, nasal aspiration and other methods. Vaccines of the "naked nucleic acid" type have been demonstrated to provoke an immunological response including generation of CTLs specific for the peptide encoded by the naked nucleic acid (*Science* 259:1745-1748, 1993).

The alt.M-CSF immunogenic polypeptide, as well as complexes of alt.M-CSF immunogenic polypeptide and HLA molecule, also may be used to produce antibodies, using standard techniques well known to the art. Standard reference works setting forth the general principles of antibody production include Catty, D., Antibodies, A Practical Approach, Vol. 1, IRL Press, Washington DC (1988); Klein, J., Immunology: The Science of Cell-Non-Cell Discrimination, John Wiley and Sons, New York (1982); Kennett, R., et al., Monoclonal Antibodies, Hybridoma, A New Dimension In Biological Analyses, Plenum Press, New York (1980); Campbell, A., Monoclonal Antibody Technology, in Laboratory Techniques and Biochemistry and Molecular Biology, Vol. 13 (Burdon, R. et al. EDS.), Elsevier Amsterdam (1984); and Eisen, H.N., Microbiology, third edition, Davis, B.D. et al. EDS. (Harper & Rowe, Philadelphia (1980).

The antibodies of the present invention thus are prepared by any of a variety of methods, including administering protein, fragments of protein, cells expressing the protein or

fragments thereof and an appropriate HLA class I molecule, and the like to an animal to induce polyclonal antibodies. The production of monoclonal antibodies is according to techniques well known in the art. As detailed herein, such antibodies may be used for example to identify tissues expressing protein or to purify protein. Antibodies also may be coupled to specific labeling agents for imaging or to antitumor agents, including, but not limited to, methotrexate, radioiodinated compounds, toxins such as ricin, other cytostatic or cytolytic drugs, and so forth. Antibodies prepared according to the invention also preferably are specific for the peptide/HLA complexes described herein.

When "disorder" or "condition" is used herein, it refers to any pathological condition where the alt.M-CSF immunogenic polypeptide is expressed. Such disorders include cancers, such as renal cell carcinomas, etc.

Some therapeutic approaches based upon the disclosure are premised on inducing a response by a subject's immune system to alt.M-CSF immunogenic polypeptide presenting cells. One such approach is the administration of autologous $CD8^+$ T cells specific to the complex of alt.M-CSF immunogenic polypeptide and an HLA class I molecule to a subject with abnormal cells of the phenotype at issue. It is within the skill of the artisan to develop such $CD8^+$ T cells *in vitro*. Generally, a sample of cells taken from a subject, such as blood cells, are contacted with a cell presenting the complex and capable of provoking $CD8^+$ T lymphocytes to proliferate. The target cell can be a transfectant, such as a transfected COS cell, or a transfected antigen presenting cell bearing HLA class I molecules, such as dendritic cells or B cells. These transfectants present the desired complex of their surface and, when combined with a $CD8^+$ T lymphocyte of interest, stimulate its proliferation. COS cells, are widely available, as are other suitable host cells. The clonally expanded autologous $CD8^+$ T lymphocytes then are administered to the subject. The $CD8^+$ T lymphocytes then stimulate the subject's immune response, thereby achieving the desired therapeutic goal.

Another method for selecting antigen-specific CTL clones has recently been described (Altman et al., *Science* 274:94-96, 1996; Dunbar et al., *Curr. Biol.* 8:413-416, 1998), in which fluorogenic tetramers of MHC class I molecule/peptide complexes are used to detect specific CTL clones. Briefly, soluble MHC class I molecules are folded *in vitro* in the presence of β_2-microglobulin and a peptide antigen which binds the class I molecule. After purification, the MHC/peptide complex is purified and labeled with biotin. Tetramers are formed by mixing the biotinylated peptide-MHC complex with labeled avidin (e.g. phycoerythrin) at a molar

ratio or 4:1. Tetramers are then contacted with a source of CTLs such as peripheral blood or lymph node. The tetramers bind CTLs which recognize the peptide antigen/MHC class I complex. Cells bound by the tetramers can be sorted by fluorescence activated cell sorting to isolate the reactive CTLs. The isolated CTLs then can be expanded *in vitro* for use as described herein.

To detail a therapeutic methodology, referred to as adoptive transfer (Greenberg, *J. Immunol.* 136(5): 1917, 1986; Riddel et al., *Science* 257: 238, 1992; Lynch et al, *Eur. J. Immunol.* 21: 1403-1410, 1991; Kast et al., *Cell* 59: 603-614, 1989), cells presenting the desired complex are combined with CTLs leading to proliferation of the CTLs specific thereto. The proliferated CTLs are then administered to a subject with a cellular abnormality which is characterized by certain of the abnormal cells presenting the particular complex. The CTLs then lyse the abnormal cells, thereby achieving the desired therapeutic goal.

The foregoing therapy assumes that at least some of the subject's abnormal cells present the relevant HLA/TRA complex. This can be determined very easily, as the art is very familiar with methods for identifying cells which present a particular HLA molecule, as well as how to identify cells expressing DNA of the pertinent sequences, in this case a tumor associated gene sequence. Once cells presenting the relevant complex are identified via the foregoing screening methodology, they can be combined with a sample from a patient, where the sample contains CTLs. If the complex presenting cells are lysed by the mixed CTL sample, then it can be assumed that a tumor associated gene derived TRA is being presented, and the subject is an appropriate candidate for the therapeutic approaches set forth *supra.*

Adoptive transfer is not the only form of therapy that is available in accordance with the invention. $CD8^+$ T lymphocytes can also be provoked *in vivo*, using a number of approaches. One approach is the use of non-proliferative cells expressing the complex. The cells used in this approach may be those that normally express the complex, which could be dendritic cells or cells transfected with one or both of the genes necessary for presentation of the complex. Chen et al., (*Proc. Natl. Acad. Sci. USA* 88: 110-114, 1991) exemplifies this approach, showing the use of transfected cells expressing HPV-E7 peptides in a therapeutic regime. Various cell types may be used. Similarly, vectors carrying one or both of the genes of interest may be used. Viral or bacterial vectors are especially preferred. For example, nucleic acids which encode an alt.M-CSF immunogenic polypeptide may be operably linked to promoter and enhancer sequences which direct expresion of the alt.M-CSF immunogenic

polypeptide in certain tissues or cell types. The nucleic acid may be incorporated into an expression vector. Expression vectors may be unmodified extrachromosomal nucleic acids, plasmids or viral genomes constructed or modified to enable insertion of exogenous nucleic acids, such as those encoding alt.M-CSF immunogenic polypeptides. Nucleic acids encoding an alt.M-CSF immunogenic polypeptide also may be inserted into a retroviral genome, thereby facilitating integration of the nucleic acid into the genome of the target tissue or cell type. In these systems, the gene of interest is carried by a microorganism, e.g., a Vaccinia virus, retrovirus or the bacteria BCG, and the materials de facto "infect" host cells. The cells which result present the complex of interest, and are recognized by autologous $CD8^+$ T cells, which then proliferate.

A similar effect can be achieved by combining an alt.M-CSF immunogenic polypeptide with an adjuvant to facilitate incorporation into HLA class I presenting cells *in vivo*. If larger than the HLA class I binding portion, the alt.M-CSF immunogenic polypeptide can be processed if necessary to yield the peptide partner of the HLA molecule while the TRA is presented without the need for further processing. Generally, subjects can receive an intradermal injection of an effective amount of the alt.M-CSF immunogenic polypeptide. Initial doses can be followed by booster doses, following immunization protocols standard in the art.

As part of the immunization protocols, substances which potentiate the immune response may be administered with nucleic acid or peptide components of a cancer vaccine. Such immune response potentiating compound may be classified as either adjuvants or cytokines. Adjuvants may enhance the immunological response by providing a reservoir of antigen (extracellularly or within macrophages), activating macrophages and stimulating specific sets of lymphocytes. Adjuvants of many kinds are well known in the art; specific examples include MPL (SmithKline Beecham), a congener obtained after purification and acid hydrolysis of *Salmonella minnesota* Re 595 lipopolysaccharide, QS21 (SmithKline Beecham), a pure QA-21 saponin purified from *Quillja saponaria* extract, DQS21, described in PCT application WO96/33739 (SmithKline Beecham), vitamin E and various water-in-oil emulsions prepared from biodegradable oils such as squalene and/or tocopherol. Cytokines are also useful in vaccination protocols as a result of lymphocyte stimulatory properties. Many cytokines useful for such purposes will be known to one of ordinary skill in the art, including interleukin-12 (IL-12) which has been shown to enhance the protective effects of

vaccines (*Science* 268: 1432-1434, 1995), GM-CSF and IL-18.

There are a number of additional immune response potentiating compounds that can be used in vaccination protocols. These include costimulatory molecules provided in either protein or nucleic acid form. Such costimulatory molecules include the B7-1 and B7-2 (CD80 and CD86 respectively) molecules which are expressed on dendritic cells (DC) and interact with the CD28 molecule expressed on the T cell. This interaction provides costimulation (signal 2) to an antigen/MHC/TCR stimulated (signal 1) T cell, increasing T cell proliferation and effector function. B7 also interacts with CTLA4 (CD152) on T cells and studies involving CTLA4 and B7 ligands indicate that the B7-CTLA4 interaction can enhance antitumor immunity and CTL proliferation (Zheng et al., *Proc. Nat'l Acad. Sci. USA* 95:6284-6289, 1998).

B7 typically is not expressed on tumor cells so they are not efficient antigen presenting cells (APCs) for T cells. Induction of B7 expression would enable the tumor cells to stimulate more efficiently CTL proliferation and effector function. A combination of B7/IL-6/IL-12 costimulation has been shown to induce IFN-gamma and a Th1 cytokine profile in the T cell population leading to further enhanced T cell activity (Gajewski et al., *J. Immunol.* 154:5637-5648, 1995). Tumor cell transfection with B7 has been discussed in relation to *in vitro* CTL expansion for adoptive transfer immunotherapy by Wang et al. (*J. Immunother.* 19:1-8, 1996). Other delivery mechanisms for the B7 molecule would include nucleic acid (naked DNA) immunization (Kim et al., *Nature Biotechnol.* 15:7:641-646, 1997) and recombinant viruses such as adeno and pox (Wendtner et al., *Gene Ther.* 4:726-735, 1997). These systems are all amenable to the construction and use of expression cassettes for the coexpression of B7 with other molecules of choice such as the antigens or fragment(s) of antigens discussed herein (including polytopes) or cytokines. These delivery systems can be used for induction of the appropriate molecules *in vitro* and for *in vivo* vaccination situations. The use of anti-CD28 antibodies to directly stimulate T cells *in vitro* and *in vivo* could also be considered. Similarly, the inducible co-stimulatory molecule ICOS which induces T cell responses to foreign antigen could be modulated, for example, by use of anti-ICOS antibodies (Hutloff et al., *Nature* 397:263-266, 1999).

Lymphocyte function associated antigen-3 (LFA-3) is expressed on APCs and some tumor cells and interacts with CD2 expressed on T cells. This interaction induces T cell IL-2 and IFN-gamma production and can thus complement but not substitute, the B7/CD28

costimulatory interaction (Parra et al., *J. Immunol.*, 158:637-642, 1997; Fenton et al., *J. Immunother.*, 21:95-108, 1998).

Lymphocyte function associated antigen-1 (LFA-1) is expressed on leukocytes and interacts with ICAM-1 expressed on APCs and some tumor cells. This interaction induces T cell IL-2 and IFN-gamma production and can thus complement but not substitute, the B7/CD28 costimulatory interaction (Fenton et al., 1998). LFA-1 is thus a further example of a costimulatory molecule that could be provided in a vaccination protocol in the various ways discussed above for B7.

Complete CTL activation and effector function requires Th cell help through the interaction between the Th cell CD40L (CD40 ligand) molecule and the CD40 molecule expressed by DCs (Ridge et al., *Nature* 393:474, 1998; Bennett et al., *Nature* 393:478, 1998; Schoenberger et al., *Nature* 393:480, 1998). This mechanism of this costimulatory signal is likely to involve upregulation of B7 and associated IL-6/IL-12 production by the DC (APC). The CD40-CD40L interaction thus complements the signal 1 (antigen/MHC-TCR) and signal 2 (B7-CD28) interactions.

The use of anti-CD40 antibodies to stimulate DC cells directly, would be expected to enhance a response to tumor associated antigens which are normally encountered outside of an inflammatory context or are presented by non-professional APCs (tumor cells). In these situations Th help and B7 costimulation signals are not provided. This mechanism might be used in the context of antigen pulsed DC based therapies or in situations where Th epitopes have not been defined within known tumor associated antigen precursors.

When administered, the therapeutic compositions of the present invention are administered in pharmaceutically acceptable preparations. Such preparations may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, supplementary immune potentiating agents such as adjuvants and cytokines and optionally other therapeutic agents. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. The characteristics of the carrier will depend on the route of administration.

The therapeutics of the invention can be administered by any conventional route, including injection or by gradual infusion over time. The administration may, for example, be oral, intravenous, intraperitoneal, intramuscular, intracavity, subcutaneous, intradermal or

transdermal.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

The invention also contemplates gene therapy. The procedure for performing *ex vivo* gene therapy is outlined in U.S. Patent 5,399,346 and in exhibits submitted in the file history of that patent, all of which are publicly available documents. In general, it involves introduction *in vitro* of a functional copy of a gene into a cell(s) of a subject which contains a defective copy of the gene, and returning the genetically engineered cell(s) to the subject. The functional copy of the gene is under operable control of regulatory elements which permit expression of the gene in the genetically engineered cell(s). Numerous transfection and transduction techniques as well as appropriate expression vectors are well known to those of ordinary skill in the art, some of which are described in PCT application WO95/00654. *In vivo* gene therapy using vectors such as adenovirus also is contemplated according to the invention.

The preparations of the invention are administered in effective amounts. An effective amount is that amount of a pharmaceutical preparation that alone, or together with further doses, stimulates the desired response. In the case of treating cancer, the desired response is inhibiting the progression of the cancer. This may involve only slowing the progression of the disease temporarily, although more preferably, it involves halting the progression of the disease permanently. In the case of inducing an immune response, the desired response is an increase in antibodies or T lymphocytes which are specific for the alt.M-CSF immunogen(s) employed. These desired responses can be monitored by routine methods or can be monitored according to diagnostic methods of the invention discussed herein.

Where it is desired to stimulate an immune response using a therapeutic composition

of the invention, this may involve the stimulation of a humoral antibody response resulting in an increase in antibody titer in serum, a clonal expansion of cytotoxic lymphocytes, or some other desirable immunologic response. It is believed that doses of immunogens ranging from one nanogram/kilogram to 100 miligrams/kilogram, depending upon the mode of administration, would be effective. The preferred range is believed to be between 500 nanograms and 500 micrograms per kilogram. The absolute amount will depend upon a variety of factors, including the material selected for administration, whether the administration is in single or multiple doses, and individual patient parameters including age, physical condition, size, weight, and the stage of the disease. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation.

Examples

Methods and Materials

Cell lines

Renal cell carcinoma line LB1047-RCC was derived from a metastatic lymph node lesion of patient LB1047 (HLA-A*0201, -A*2401, -B*3501, -B51, -Cw*0401, -Cw*1602). The caryotypic analysis of the cell line performed at passage 2 showed only abnormal mitosis, confirming that cell line LB1047 was a tumor line. The lymphoblastoid cell line LB1047-EBV was derived from culture supernatant lymphocytes of the primary tumor cell culture of patient LB1047 with 1 μg/ml cyclosporin A (Sandoz, Basel, Switzerland), and 20% (v/v) of supernatant of EBV-transformed B95-8 cells using standard techniques. MER190-Mel is a melanoma cell line; MiaPaCa-2 is a pancreatic carcinoma line from ATCC. All cell lines were cultured in a 8% CO_2 incubator in Iscove's medium containing 10% FCS, except LE9211-EBV that was grown in RPMI 1640 in a 5% CO_2 incubator; all media were supplemented with L-arginine (116 mg/ml), L-asparagine (36 mg/ml), L-glutamine (216 mg/ml), penicillin (200 U/ml), and streptomycin (100μg/ml).

Short term cultures of normal renal tissue

Normal renal tissue was obtained from patients LB2046 and LB2043, that were

operated on because of anatomical abnormalities and for renal cell carcinoma, respectively. Tissue samples were dissected with a scalpel and run through a 1 mm^2 metal mesh. The culture medium was Iscove's medium as described above, but supplemented further with insulin (20 μg/ml), transferrin (10 μg/ml), sodium selenite (25 nM), hydrocortisone (50 nM), ethanolamine (10 μM), phosphorylethanolamine (10 μM), triidoo-L-thyroxine (100 pM), bovine serum albumin (2 mg/ml), epidermal growth factor (1 ng/ml), and sodium pyruvate (0.5 mM) (Gazdar, 1986).

Antitumor CTL clones

The culture supernatant lymphocytes from the primary tumor culture of LB1047-RCC were collected at day 3, expanded for one week with IL-6 (10^3 U/ml) and IL-12 (10 ng/ml), and frozen at -80°C until establishment of the autologous tumor cell line. An autologous mixed lymphocyte tumor cell culture (MLTC) was performed as described previously with minor modifications (Herin et al., *Int. J. Cancer* 39:390-396, 1987). Stimulator cells were treated with IFN-γ for 24 h before the stimulation. The MLTC was performed by mixing 1 x 10^6 lymphocytes with 1 x 10^5 irradiated LB1047-RCC tumor cells culture in human serum and with IL-6 (10^3 U/ml) and IL-12 (10 ng/ml). After one week, 5 x 10^5 lymphocytes were stimulated again for one week with irradiated tumor cells (1 x 10^5) with IL-7 (5 ng/ml) and IL-2 (10 U/ml), and then one week further on with tumor cells and IL-2 (25 U/ml). On day 21, lymphocytes from the culture were cloned by limited dilution in Iscove's medium supplemented with IL-1 (50 U/ml), and long-term culture of CTL clones was performed as described (Herin, 1987).

Cytotoxicity assay

The lytic activity of CTL clones were tested in a standard chromium release assay as described (Boon et al., *J. Exp. Med.* 152(5):1184-1193, 1980). Where indicated, target cells were treated with 100 U/ml IFN-γ 24 h before the assay. Chromium release was measured after 4 h. For peptide assay, labeled EBV-transformed B-cells were incubated 30 min at 37°C with various concentrations of peptides diluted in X-vivo 10 medium (Whittaker Bioproducts, Walkersville MD).

CTL stimulation assay

A total of 2500-10000 CTLs (depending on the test) were added to microwells containing stimulator cells in 100 μl of Iscove's medium supplemented with 10% pooled human serum and 25 U/ml of IL-2. The supernatant was collected after 24 h, and TNF or IFN-γ was determined. TNF was detected by testing the cytotoxic effect on WEHI-164 clone 13 cells (Espevik and Nissen-Meyer, *J. Immunol. Methods*, 95:99-105, 1986) in a 3-(4,5-dimethylthiazol-2-yl-)-2,5,-diphenyl tetrazolium bromide MTT colorimetric assay (Hanson *et al., J. Immunol. Methods*, 119:203-210, 1989). IFN-γ was detected using a commercial quantitative sandwich immunoassay kit from BioSource. Inhibition with W6/32, B1-23-2 (anti-HLA-B and -C), and C7709A.2.6 (anti-HLA-A24) mAbs was performed by adding a 1/20 dilution of ascites to the test.

M-CSF detection

M-CSF was detected in culture supernatants of 1 x 10^5 cells plated in 0.5 ml of medium in 48-well microtiter plated after 24 hr using a commercial quantitative sandwich immunoassay kit (R & D Systems). For detection of M-CSF in cryosections a mouse IgG2A mAb against human M-CSF (clone 26730.11; R & D Systems) was used. As a detection system the DAKO EnVision™+ AEC-system (DAKO Corporation, Carpinteria CA) was used. All steps were done at room temperature. For counterstaining, haematoxylin (Merck) was used.

Immunohistochemistry

Frozen tissue sections (7 μm thickness, fixed for 5 min in formol) or paraffin-embedded histological specimen material (5μm thickness) were washed in Tris 50 mM, pH7.4, NaCl 150 mM (TBS). Endogenous peroxidase activity was blocked for 10 min with H_2O_2. Goat IgG (Sigma) at 0.5 mg/ml diluted in 0.5% (w/v) BSA/PBS was used to block non-specific Ig-binding sites for 30 min. Incubation with primary antibody or control antibody was done thereafter for 1 hour. Detection and counterstaining were performed as described above.

Example 1: Cloning of CTLs responsive to renal cell carcinoma (RCC)

Renal cell carcinoma line LB1047-RCC is derived from a clear cell carcinoma that contained many infiltrating lymphocytes. Therefore, at day 3 of the primary tumor culture,

these tumor-infiltrating lymphocytes (TIL) were collected from the culture supernatant, expanded for one week with IL-6 (10^3 U/ml) and IL-12 (10 ng/ml), and frozen after one week of culture until establishment of the autologous tumor cell line, which was completed about one month later.

The mixed lymphocyte tumor-cell culture (MLTC 403) was then performed as described above. A number of clones were obtained including CTLs 403A/9, 403B/8 and 403A/2. CTL 403A/9 recognized and lysed the autologous tumor line LB1047-RCC and the allogeneic renal cell carcinoma line LE9211-RCC (HLA-B35 positive) but not the corresponding allogenic EBV-transformed B-cell line LE9211-EBV or NK-target K562 cells (Fig. 1). Autologous EBV-transformed B-cells were not available because the patient died before blood lymphocytes could be collected. Where indicated, LB1047-RCC cells were treated with 100 U/ml IFN-γ 24 hours before the test to increase to expression of HLA-class I. Chromium release was measured after 4 hours.

CTL 403A/9 produced IFN-γ when stimulated with LB1047-RCC cells, and this production was inhibited by anti-class I antibody W6/32 (Fig. 2) and by anti-B/C antibody (not shown). The restriction element was either HLA-B35 or HLA-Cw4, because these were the only HLA-class I molecules shared by LB1047-RCC and LE9211-RCC.

CTL 403B/8 was also inhibited by anti-HLA-B or -C reactive mAb, but recognized different allogeneic tumor cell lines that share the restriction element HLA-Cw4.

CTL 403A/2 was HLA-A restricted, because cytokine secretion was not inhibited by anti-HLA-B or -C reactive mAb. Allogeneic tumor cell lines that share the restriction element HLA-A24 also stimulated CTL 403A/2, and induction of TNF was totally blocked by a mAb recognizing HLA-A24; therefore this CTL clone was restricted by HLA-A24.

Example 2: Identification of the antigen recognized by RCC-specific CTLs

To identify the antigen, an oligo-dT-based cDNA library was constructed from LB1047-RCC cells as described recently (Guéguen, et al., *J. Immunol.* 160:6188-6194, 1998). In brief, poly-(A)+ RNA was transcribed to cDNA with the Superscript Choice System (Life Technologies) using an oligo(dT) primer containing a Not I site at its 5' end [5'-ATAAGAATGCGGCCGCTAAACTATTTTTTTTTTTTTTTTT(A/G/C)(A/G/C/T)-3'; SEQ ID NO:1]. The cDNA was ligated to Hind III adaptors (Stratagene, La Jolla, CA), phosphorylated, digested with Not I, and inserted into the Hind III and Not I sites of the

expression vector pCEP4 (Invitrogen, Carlsbad, CA). DNA from pools of about 100 cDNAs from this library were co-transfected into 293-EBNA cells as described by Guéguen, et al., 1998. Briefly, 5 x 10^4 293-EBNA cells were plated in flat-bottom 96-well plates and transfected with 60 ng each of both the HLA-B*3501 and the HLA-Cw4 cDNAs and about 100 ng of plasmid DNA from a pool of the cDNA library. The cDNA of the autologous HLA-B*3501 and HLA-Cw*0401 were cloned before by PCR from cDNA. For HLA-B35 amplification HLA-class I specific primers were used according to Ennis et al. (*Proc. Natl. Acad. Sci. U.S.A.* 87:2833-2837, 1990); PCR products were digested thereafter with Kpn I to eliminate HLA-A sequences, phosphorylated, and inserted into the EcoR V site of pcDNA3. HLA-Cw*0401 was amplified with HLA-C-specific primers containing a BamH I and EcoR I site in the sense and antisense primers, respectively [sense primer 5'-CGGGATCCGCCGAGATGCGGGTCAT-3', SEQ ID NO:2; reverse primer 5'-CGGAATTCTCAGGCTTTACAAGCGATGAGA-3', SEQ ID NO:3]. The PCR products were digested with EcoR I and BamH I and inserted into pcDNA3. HLA-B*3501 and HLA-Cw*0401 were both sequenced, to verify their identity.

Because the tumor cells produced significant amounts of TNF, the transfected 293-EBNA cells were screened for their ability to stimulate CTL 403A/9 by measuring its IFN-γ secretion by ELISA (Biosource, Camarillo, CA) instead of TNF (Fig. 2). CTL 403A/9 were stimulated with 293-EBNA cells transfected with indicated cDNAs or with autologous tumor cells in the presence of the indicated antibody. Because HLA-B35 and HLA-Cw4 were the possible restriction elements for CTL 403A/9, pool screening was done by transfecting both HLA molecules together. IFN-γ was measured after 24 hours. Pool D77 proved positive and was subcloned.

In the experiments of Fig. 3, CTL 403A/9 was stimulated with autologous tumor cells or with 293-EBNA cells transfected with indicated cDNAs. IFN-γ was measured after 24 hours. From pool D77, two cDNAs were isolated that were able to stimulate CTL 403A/9 when transfected with the HLA-B*3501 cDNA (pD77-314 and pD77-344; Fig. 3).

The sequence of both cDNAs corresponded to that of the 4 kb splice variant of the human macrophage colony-stimulating factor (M-CSF; Roth and Stanley, *Curr. Topics Microbiol. Immunol.* 181:143-167, 1992). This gene appears to be strongly overexpressed in renal cell carcinoma (Reinecke, et al. *Eur. J. Cancer* 33(suppl.8):S39, 1997).

To identify the region coding for the antigenic peptide, truncated variants of pD77-314

obtained by exonuclease III digestions were tested. The antigenic peptide was localized to the 5' end of the M-CSF cDNA. A series of synthetic peptides corresponding to this region of the M-CSF protein sequence were tested, but none of them was recognized by CTL 403A/9. Putative proteins encoded by the 5' end of the M-CSF cDNA were searched for in the other two reading frames. An alternative open reading frame (SEQ ID NO:4) was identified which codes for a putative protein of 25 amino acids (alt.M-CSF; SEQ ID NO:5) (Fig. 4). Minigene MPO29/30, which encodes this alt.M-CSF, was then constructed by PCR using sense primer MPO29 (5'-ACTGGGCGGATCCTGCCCTCCCACGACATGGCT-3'; SEQ ID NO:6) and antisense primer MPO30 (5'-ACTGCCCGAATTCGTCACGAGGTCTCCATCTGACTG-3'; SEQ ID NO:7), which contain a BamH I and EcoR I site, respectively. The PCR product was digested with BamH I and EcoR I, inserted into pcDNA3, and sequenced, to verify its identity. This minigene stimulated the CTL when transfected with the HLA-B*3501 cDNA, suggesting that the antigenic peptide was not derived from the normal M-CSF protein but from this alt.M-CSF (Fig. 5). Fig. 5 shows that CTL 403/9 was stimulated with autologous tumor cells or with 293-EBNA cells transfected with indicated cDNAs. IFNγ was measured after 24 hours.

To further define the antigenic peptide, another minigene (MPO29/39) was constructed encoding a truncated form of alt.M-CSF containing the 20 first amino acids (SEQ ID NO:9; the nucleotide sequence encoding the 20-mer is SEQ ID NO:8), using sense primer MPO29 and antisense primer MPO39 (5'-ACTGCCCGAATTCTATCCTC GGTGATACTCCTG-3'; SEQ ID NO:10) (Fig. 4). This minigene also proved positive when transfected with the HLA-B*3501 cDNA and tested with CTL 403A/9 (Fig. 5). The antigenic peptide is therefore included within the 20 first amino acids.

Several synthetic peptides corresponding to the sequence of this alt.M-CSF were then tested and it was found that peptide LPAVVGLSPGEQEY (SEQ ID NO:12; the nucleotide sequence which encodes it is SEQ ID NO:11) was able to sensitize allogeneic HLA-B*3501-positive EBV-B-cells (HA7-EBV) to lysis by CTL 403A/9 (Fig. 6). Chromium-labeled HA7-EBV cells were incubated 30 min at 37°C with the indicated concentrations of peptide. CTL 403A/9 was added at an effector to target ratio of 10:1, and chromium release was measured after 4 hours. HA7-EBV cells were derived from renal carcinoma patient HA7; the corresponding tumor line HA7-RCC was established later and is also recognized by CTL 403A/9.

According to the current knowledge, a proline in position 2 and a hydrophobic residue at the C-terminus of the peptide fit with the HLA-B35 binding peptide, but the length of 14 amino acids has not been reported yet for an HLA-class I binding peptide recognized by CTL. To confirm that this 14-mer is the optimal antigenic peptide, peptides without the first or the last amino acid were tested. HA7-EBV cells were incubated 30 min at 37°C with the indicated concentrations of peptide before addition of CTL at an effector to target ratio of 0.2. The amount of IFN-γ released in the supernatant was measured after 24 hours. Peptides PAVVGLSPGEQEY (SEQ ID NO:13), LPAVVGLSPGEQE (SEQ ID NO:14) and AGLPAVVGLSPGEQE (SEQ ID NO:15) were not able to sensitize allogeneic HLA-B*3501-positive EBV-B-cells HA7-EBV (Figs. 6 and 7), while the 14-mer peptide LPAVVGLSPGEQEY (SEQ ID NO:12) did sensitize these cells. The 14-mer therefore appears to be the optimal peptide recognized by CTL 403A/9.

In summary, the antigenic peptide LPAVVGLSPGEQEY (SEQ ID NO:12) is derived from the translation of an alternative open reading frame of the normal human M-CSF cDNA, and is recognized by CTL 403A/9 on the autologous and two allogeneic HLA-B*3501 positive renal cell carcinoma lines.

A search of 10 amino acid HLA peptide motifs for the HLA-A24 and HLA-Cw4 recognition elements at the National Institutes of Health website (http://bimas.dcrt.nih.gov) indicated that amino acids 16-25 of SEQ ID NO:5 consititute a peptide which may be presented by these HLA molecules. Given the results for HLA-B*3501, the optimal peptides for HLA-A24 and HLA-Cw4 may be a different size or sequence, which can be determined as described above. This and other alt.M-CSF peptides can be synthesized and tested for HLA binding as described above for HLA-B*3501.

Example 3: Expression of alt.M-CSF in normal and tumor tissues

Expression of the major M-CSF product is mainly regulated at the posttranscriptional level, and observed in areas of tissue inflammation, or in the human placenta where it functions as a growth and development factor. Constitutive overexpression of M-CSF product is frequent in cancers of the urogenital tract, such as renal, bladder, and prostate or breast, ovarian and uterine tumors measured either by immunohistochemistry or by ELISA of cell culture supernatants.

Expression of the M-CSF was analyzed by RT-PCR in normal and tumor cells. No

correlation was found between the presence of M-CSF mRNA in a cell and recognition of the cell by CTL 403A/9. This result suggests that the expression of alt.M-CSF antigenic peptide precursor is controlled by translation of the alt.M-CSF ORF, which may be translated independently from the major ORF encoding M-CSF.

To investigate the tumor specificity of the alt.M-CSF product, a polyclonal rabbit serum was raised against a HPLC-purified synthetic peptide of 25 amino acids corresponding to alt.M-CSF (SEQ ID NO:5) coupled to KLH (EUROGENTEC, Ougree, Belgium). The polyclonal antiserum was purified by an affinity column made by coupling the 25 amino acid alt.M-CSF product to CNBr-activated Sepharose 4B as directed by the manufacturer (Pharmacia Biotech).

To assess the expression in primary tumor samples, the expression of alt.M-CSF was first examined by immunohistochemistry in paraffin sections of different kidney tumors. Six of 10 renal cell carcinomas expressed alt.M-CSF including the autologous tumor LB1047-RCC specimen. Expression of alt.M-CSF was also detected in normal tubular cells of patient LB1047; the expression of alt.M-CSF in normal tubular cells was confirmed in different kidney sections. Paraffin sections of a kidney donor biopsy were stained with either rabbit polyclonal alt.M-CSF antiserum or rabbit control IgG. Confirmation of the specificity of alt.M-CSF staining was done by peptide inhibition with either a mixture of 15mers of the alt.M-CSF product (amino acids 1-15, 6-20, 11-25 of SEQ ID NO:5) or a control M-CSF 17mer peptide.

To determine the expression of M-CSF in normal tubular kidney cells, cryosections of patient LB2043 or a renal cell carcinoma sample were stained with either anti-human M-CSF mAb or anti-melanoma mAb KBA-2 isotype control. The major M-CSF product was not detectable in normal tubular kidney cells *in vivo* Because M-CSF is not constitutively expressed in these normal tubular kidney cells, the translation of alt.M-CSF is regulated independently of M-CSF.

To test the recognition of normal tubular kidney cells by CTL 403A/9, short term kidney cell cultures of patients LB2046 and LB2043 were used. These short term cultures were mainly of proximal tubular cell origin (PTEC) as determined by the expression of aquaporin. Because the HLA typing of the short term culture lines was unknown, HLA-B*3501 was transfected into LB2046-PTEC and LB2043-PTEC. Expression of HLA-B35 was confirmed by pulsing the specific peptide recognized by CTL403A/9 on transfected and

non-transfected stimulator cells. TNF was measured after 24 h. Both PTEC lines stimulate TNF release in CTL 403A/9. The corresponding M-CSF levels in the supernatant of stimulator cells was assessed by ELISA after 24h. The proximal tubular kidney cell cultures both stimulated TNF production in CTL 403A/9 similar to the autologous tumor LB1047-RCC; whereas LB2046 expressed HLA-B*3501 itself and stimulated CTL 403A/9 similar to the autologous tumor LB1047-RCC, LB2043-PTEC was recognized only after transient transfection of HLA-B*3501 (Fig. 8). Contrary to the *in vivo* situation, these short term PTEC lines were both activated to secrete M-CSF (Fig. 8).

M-CSF secretion of stimulator cells and alt.M-CSF expression as assessed by IFN-γ induction in CTL 403A/9 were correlated (Fig. 9). MER190-Mel secreted only 99 pg/ml of M-CSF compared to 858 pg/ml of MiaPaCa-2 cells stably transfected with HLA-B35, or more than 4000 pg/ml for RCC line LE9211-RCC as depicted on the right side of Fig. 9. IFN-γ was measured after 24 h. There is no correlation of the amount of IFN-γ induction in CTL 403A/9 and M-CSF secretion of the respective stimulator cells. Therefore, the translation of alt.M-CSF is independent of M-CSF translation.

Therefore, because of the expression in normal kidney cells and because the expression is independently regulated from M-CSF itself, it can be concluded that the alt.M-CSF product is not expressed exclusively in tumor cells.

Expression of the alt.M-CSF product in other normal tissues was investigated because of the constitutive expression of alt.M-CSF in normal renal tubular cells. It was determined that alt.M-CSF was also expressed in normal hepatocytes, but not in normal thyroid gland, or in gastric, colon, placenta, ovary or breast tissue.

EQUIVALENTS:

Other aspects of the invention will be clear to the skilled artisan and need not be repeated here. Each reference cited herein is incorporated by reference in its entirety.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

We claim:

Claims

1. An isolated polypeptide comprising the amino acid sequence of SEQ ID NO:5, or a fragment thereof having at least 14 consecutive amino acids of SEQ ID NO:5.

2. An isolated immunogenic polypeptide comprising the amino acid sequence of SEQ ID NO:12, or a functional variant thereof.

3. The isolated immunogenic polypeptide of claim 2 wherein the isolated immunogenic peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:9 and SEQ ID NO:12.

4. The isolated immunogenic polypeptide of claim 1 wherein the isolated polypeptide consists of the amino acid sequence of SEQ ID NO:12.

5. The isolated polypeptide of claim 1 or claim 2 wherein the isolated polypeptide is non-hydrolyzable.

6. The isolated polypeptide of claim 5 wherein the isolated polypeptide is selected from the group consisting of peptides comprising D-amino acids, peptides comprising a -psi[CH_2NH]-reduced amide peptide bond, peptides comprising a -psi[$COCH_2$]-ketomethylene peptide bond, peptides comprising a -psi[CH(CN)NH]-(cyanomethylene)amino peptide bond, peptides comprising a -psi[$CH_2CH(OH)$]-hydroxyethylene peptide bond, peptides comprising a -psi[CH_2O]-peptide bond, and peptides comprising a -psi[CH_2S]-thiomethylene peptide bond.

7. A composition comprising the isolated immunogenic polypeptide of claim 2.

8. The composition of claim 7, further comprising an isolated non-alt.M-CSF tumor rejection antigen peptide or a precursor thereof.

9. The composition of claim 7, wherein the isolated immunogenic polypeptide comprises the amino acid sequence of SEQ ID NO:12.

10. The composition of claim 9, wherein the isolated immunogenic polypeptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:9, and SEQ ID NO:12.

11. An isolated nucleic acid encoding a peptide selected from the group consisting of the polypeptide of claim 1 and the immunogenic polypeptide of claim 2, wherein the isolated nucleic acid, when translated in frame, does not encode M-CSF, a precursor of M-CSF, or a fragment of M-CSF.

12. The isolated nucleic acid of claim 11, wherein the nucleic acid comprises SEQ ID NO:11.

13. An expression vector comprising the isolated nucleic acid of claim 11 operably linked to a promoter.

14. The expression vector of claim 13 wherein the nucleic acid comprises SEQ ID NO:11.

15. The expression vector of claims 13 or 14 further comprising a nucleic acid which encodes an HLA-B*3501 molecule.

16. A host cell transfected or transformed with an expression vector selected from the group consisting of the expression vector of claim 13, the expression vector of claim 14, and the expression vector of claim 15.

17. A host cell transfected or transformed with an expression vector selected from the group of the expression vector of claim 11 and the expression vector of claim 12, and wherein the host cell expresses an HLA-B*3501 molecule.

18. A method for enriching selectively a population of T lymphocytes with $CD8^+$ T lymphocytes specific for an alt.M-CSF immunogenic polypeptide comprising:

contacting an isolated population of T lymphocytes with an agent presenting a complex of the alt.M-CSF immunogenic polypeptide and an HLA class I molecule in an

amount sufficient to selectively enrich the isolated population of T lymphocytes with the $CD8^+$ T lymphocytes.

19. The method of claim 18, wherein the agent is an antigen presenting cell contacted with an alt.M-CSF immunogenic polypeptide comprising the amino acid sequence of SEQ ID NO:12.

20. The method of claim 18 or 19 wherein the HLA class I molecule is an HLA-B*3501 molecule and wherein the alt.M-CSF immunogenic polypeptide is selected from the group consisting of:

a peptide consisting of the amino acid sequence of SEQ ID NO:5, a peptide consisting of the amino acid sequence of SEQ ID NO:9, and a peptide consisting of the amino acid sequence of SEQ ID NO:12.

21. A method for diagnosing a disorder characterized by expression of an alt.M-CSF polypeptide comprising:

contacting a biological sample isolated from a subject with an agent that is specific for the alt.M-CSF polypeptide, and

determining the interaction between the agent and the alt.M-CSF polypeptide as a determination of the disorder.

22. The method of claim 21 wherein the alt.M-CSF polypeptide comprises the amino acid sequence of SEQ ID NO:12.

23. The method of claim 22, wherein the alt.M-CSF polypeptide is selected from the group consisting of:

a polypeptide consisting of the amino acid sequence of SEQ ID NO:5 and a polypeptide consisting of the amino acid sequence of SEQ ID NO:12.

24. A method for diagnosing a disorder characterized by expression of an alt.M-CSF immunogenic polypeptide which forms a complex with an HLA class I molecule, comprising:

contacting a biological sample isolated from a subject with an agent that binds the

complex; and

determining binding between the complex and the agent as a determination of the disorder.

25. The method of claim 24 wherein the HLA class I molecule is an HLA-B*3501 molecule and the alt.M-CSF immunogenic polypeptide comprises the amino acid sequence of SEQ ID NO:12.

26. The method of claim 25, wherein the alt.M-CSF immunogenic polypeptide is selected from the group consisting of:

a peptide consisting of the amino acid sequence of SEQ ID NO:5 and a peptide consisting of the amino acid sequence of SEQ ID NO:12.

27. A method for treating a subject having a disorder characterized by expression of alt.M-CSF, comprising:

administering to the subject an amount of an alt.M-CSF immunogenic polypeptide sufficient to ameliorate the disorder.

28. The method of claim 27 wherein the alt.M-CSF immunogenic polypeptide comprises the amino acid sequence of SEQ ID NO:12, or a functional variant thereof.

29. The method of claim 28, wherein the alt.M-CSF immunogenic polypeptide is selected from the group consisting of:

a peptide consisting of the amino acid sequence of SEQ ID NO:5, a peptide consisting of the amino acid sequence of SEQ ID NO:9, and a peptide consisting of the amino acid sequence of SEQ ID NO:12.

30. A method for treating a subject having a disorder characterized by expression of alt.M-CSF, comprising:

administering to the subject an amount of an agent which enriches selectively in the subject the presence of complexes of an HLA class I molecule and an alt.M-CSF immunogenic polypeptide, sufficient to ameliorate the disorder.

31. The method of claim 30 wherein the HLA class I molecule is an HLA-B*3501 molecule and the alt.M-CSF immunogenic polypeptide comprises the amino acid sequence of SEQ ID NO:12, or a functional variant thereof.

32. The method of claim 31, wherein the alt.M-CSF immunogenic polypeptide is selected from the group consisting of:

a peptide consisting of the amino acid sequence of SEQ ID NO:5, a peptide consisting of the amino acid sequence of SEQ ID NO:9, and a peptide consisting of the amino acid sequence of SEQ ID NO:12.

33. A method for treating a subject having a disorder characterized by expression of alt.M-CSF, comprising:

administering to the subject an amount of autologous $CD8^+$ T lymphocytes sufficient to ameliorate the disorder, wherein the $CD8^+$ T lymphocytes are specific for complexes of an HLA class I molecule and an alt.M-CSF immunogenic polypeptide.

34. The method of claim 33 wherein the HLA class I molecule is an HLA-B*3501 molecule and the alt.M-CSF immunogenic polypeptide comprises the amino acid sequence of SEQ ID NO:12, or a functional variant thereof.

35. The method of claim 34, wherein the alt.M-CSF immunogenic polypeptide is selected from the group consisting of:

a peptide consisting of the amino acid sequence of SEQ ID NO:5, a peptide consisting of the amino acid sequence of SEQ ID NO:9, and a peptide consisting of the amino acid sequence of SEQ ID NO:12.

36. A method for identifying functional variants of an alt.M-CSF immunogenic polypeptide, comprising

selecting an alt.M-CSF immunogenic polypeptide, an HLA class I binding molecule which binds the alt.M-CSF immunogenic polypeptide or fragment thereof, and a T lymphocyte which is stimulated by the alt.M-CSF immunogenic polypeptide or fragment thereof presented by the HLA class I binding molecule;

adding deleting or substituting a first amino acid residue of the alt.M-CSF immunogenic polypeptide to prepare a variant peptide;

determining the binding of the variant peptide to HLA class I binding molecule and the stimulation of the T lymphocyte, wherein binding of the variant peptide to the HLA class I binding molecule or stimulation of the T lymphocyte by the variant peptide presented by the HLA class I binding molecule indicates that the variant peptide is a functional variant of the alt.M-CSF immunogenic polypeptide.

37. The method of claim 36, wherein the alt.M-CSF immunogenic polypeptide comprises the amino acid sequence of SEQ ID NO:12.

38. The method of claim 36, further comprising the step of comparing the stimulation of the T lymphocyte by the alt.M-CSF immunogenic polypeptide and the stimulation of the T lymphocyte by the functional variant as a determination of the effectiveness of the stimulation of the T lymphocyte by the functional variant.

39. The method of claim 36, further comprising the step of adding, deleting or substituting at least one second amino acid to prepare a variant peptide, and

determining the binding of the variant peptide to HLA class I binding molecule and the stimulation of the T lymphocyte, wherein binding of the variant peptide to the HLA class I binding molecule or stimulation of the T lymphocyte by the variant peptide presented by the HLA class I binding molecule indicates that the variant peptide is a functional variant of the alt.M-CSF immunogenic polypeptide.

40. An isolated polypeptide which binds selectively a polypeptide of any of claims 1, 2, 3 or 4, provided that the isolated polypeptide is not an HLA class I molecule.

41. The isolated polypeptide of claim 40, wherein the isolated polypeptide is an antibody.

42. The antibody of claim 41, wherein the antibody is a monoclonal antibody.

43. The antibody of claim 41, wherein the antibody is a chimeric antibody or a humanized

antibody.

44. The isolated polypeptide of claim 40, wherein the isolated polypeptide is an antibody fragment selected from the group consisting of a Fab fragment, a $F(ab)_2$ fragment or a fragment including a CDR3 region selective for an alt.M-CSF immunogenic polypeptide.

45. An isolated $CD8^+$ T lymphocyte which selectively binds a complex of an HLA class I molecule and an alt.M-CSF immunogenic polypeptide.

46. The isolated $CD8^+$ T lymphocyte of claim 45 wherein the HLA class I molecule is an HLA-B*3501 molecule and wherein the alt.M-CSF immunogenic polypeptide comprises the amino acid sequence of SEQ ID NO:12, or a functional variant thereof.

47. The isolated $CD8^+$ T lymphocyte of claim 46 wherein the alt.M-CSF immunogenic polypeptide is selected from the group consisting of:

a peptide consisting of the amino acid sequence of SEQ ID NO:5, a peptide consisting of the amino acid sequence of SEQ ID NO:9, and a peptide consisting of the amino acid sequence of SEQ ID NO:12.

48. An isolated antigen presenting cell which comprises a complex of an HLA class I molecule and an alt.M-CSF immunogenic polypeptide.

49. The isolated antigen presenting cell of claim 48 wherein the HLA class I molecule is an HLA-B*3501 molecule and wherein the alt.M-CSF immunogenic polypeptide comprises the amino acid sequence of SEQ ID NO:12, or a functional variant thereof.

50. The isolated antigen presenting cell of claim 49 wherein the alt.M-CSF immunogenic polypeptide is selected from the group consisting of:

a peptide consisting of the amino acid sequence of SEQ ID NO:5, a peptide consisting of the amino acid sequence of SEQ ID NO:9, and a peptide consisting of the amino acid sequence of SEQ ID NO:12.

51. A method for identifying a candidate mimetic of an alt.M-CSF immunogenic polypeptide, comprising

providing a HLA molecule which binds an alt.M-CSF immunogenic polypeptide or a fragment thereof,

contacting the HLA molecule with a test molecule, and

determining the binding of the test molecule to the HLA molecule, wherein a test molecule which binds to the HLA molecule is a candidate mimetic of an alt.M-CSF immunogenic polypeptide.

52. The method of claim 51, further comprising

forming a complex of the HLA molecule and the candidate mimetic,

contacting the complex with a T cell which binds to a complex of an HLA molecule and an alt.M-CSF immunogenic polypeptide, and

assaying activation of the T cell.

53. The method of claim 52, wherein activation of the T cell is indicated by a property selected from the group consisting of proliferation of the T cell, interferon-γ production by the T cell, tumor necrosis factor production by the T cell, and cytolysis of a target cell by the T cell.

54. A vaccine composition comprising a nucleic acid encoding an alt.M-CSF immunogenic polypeptide comprising the amino acid sequence of SEQ ID NO:5 or an immunogenic fragment thereof.

55. The vaccine composition of claim 54, wherein the nucleic acid is contained in a vector selected from the group consisting of adenoviruses, adeno-associated viruses, poxviruses, vaccinia viruses, attenuated poxviruses, Semliki Forest virus, Venezuelan equine encephalitis virus, retroviruses, Sindbis virus, Ty virus-like particle and recombinant bacteria.

56. The vaccine composition of claim 54, wherein the alt.M-CSF immunogenic polypeptide or immunogenic fragment thereof comprises the amino acid sequence of SEQ ID NO:12.

57. The vaccine composition of claim 54, further comprising a nucleic acid encoding a non-alt.M-CSF immunogenic polypeptide or an immunogenic fragment thereof.

58. A vaccine composition comprising an immunogenic fragment of SEQ ID NO:5.

59. The vaccine composition of claim 58, wherein the immunogenic fragment comprises the amino acid sequence of SEQ ID NO:12.

60. The vaccine composition of claim 58, further comprising a non-alt.M-CSF immunogenic polypeptide or an immunogenic fragment thereof.

61. A vaccine composition comprising a cell which expresses an alt.M-CSF nucleic acid or polypeptide, or an immunogenic fragment thereof.

62. The vaccine composition of claim 61, the cell further comprising a non-alt.M-CSF nucleic acid or polypeptide, or an immunogenic fragment thereof.

63. The vaccine composition of any of claims 54-62, further comprising an adjuvant.

64. The vaccine composition of any of claims 54-62, further comprising a pharmaceutically acceptable carrier.

PCT

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(74) Agent: VAN AMSTERDAM, John, R.; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210 (US).

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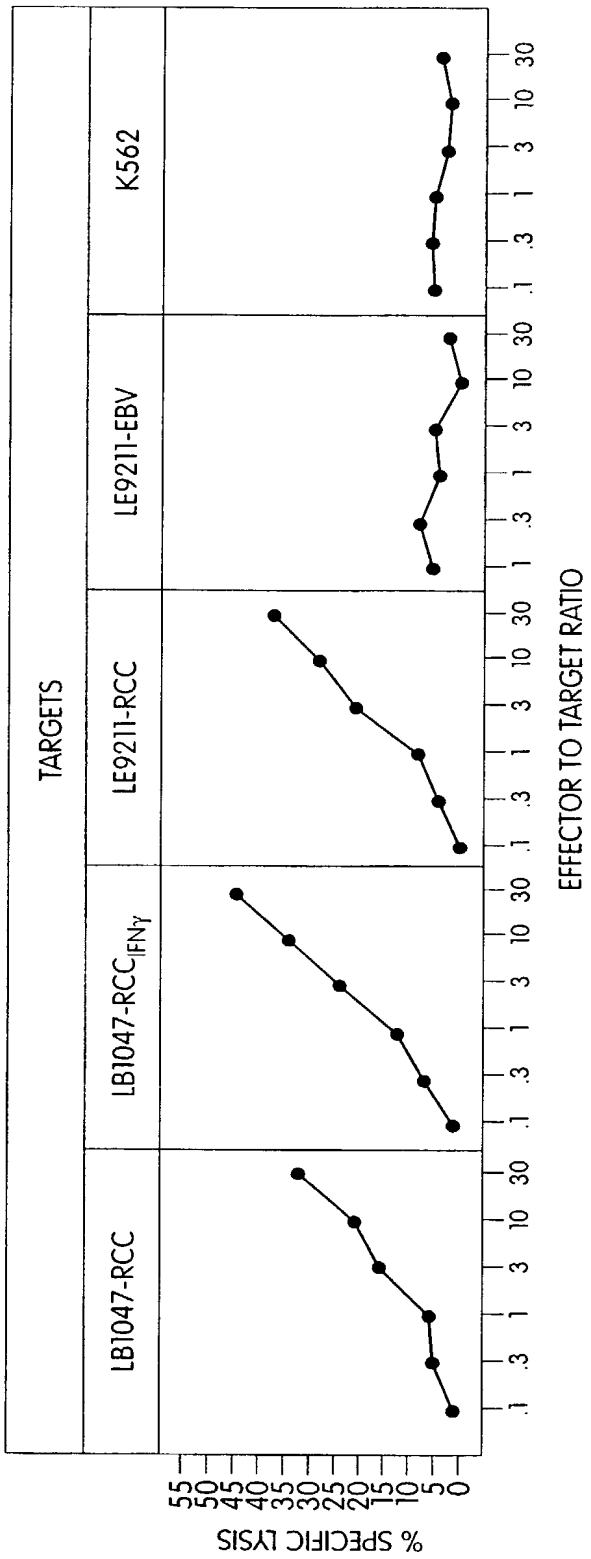
Published
With international search report.
With amended claims.

(54) Title: AN ANTIGENIC PEPTIDE ENCODED BY AN ALTERNATIVE OPEN READING FRAME OF HUMAN MACROPHAGE COLONY–STIMULATING FACTOR

(57) Abstract

The invention provides immunogenic polypeptides derived from an alternative reading frame of macrophage colony stimulating factor. Methods for diagnosis and treatment which involve the polypeptides also are provided.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LE9211-RCC
LE9211-EBV
K562
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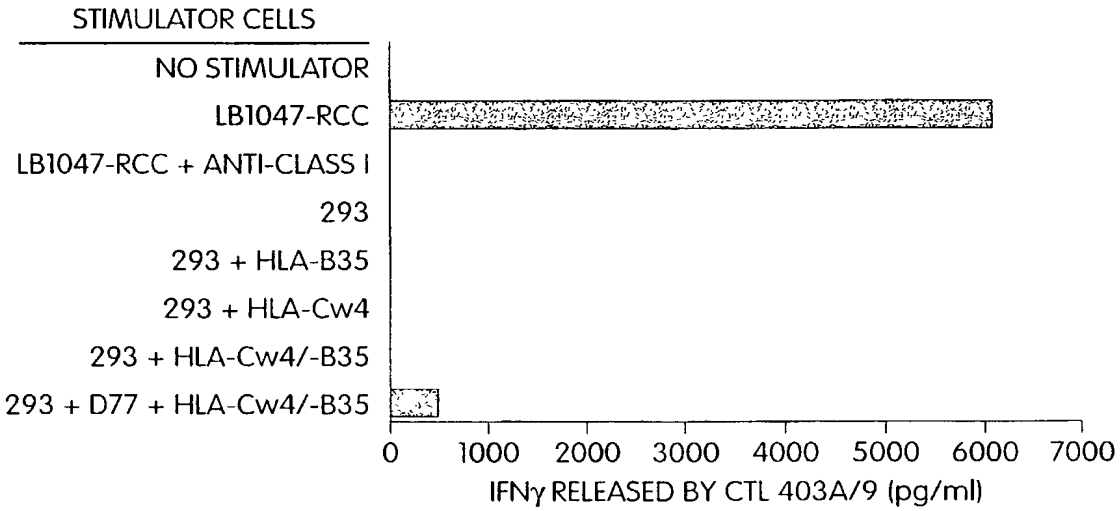
5
10
15
20
25
30
35
40
45
50
55
EFFECTOR TO TARGET RATIO
.1
.3
1
3
10
30

Fig. 1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293 + HLA-B35
293 + HLA-Cw4
293 + HLA-Cw4/-B35
293 + D77 + HLA-Cw4/-B35
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3000
4000
5000
6000
7000
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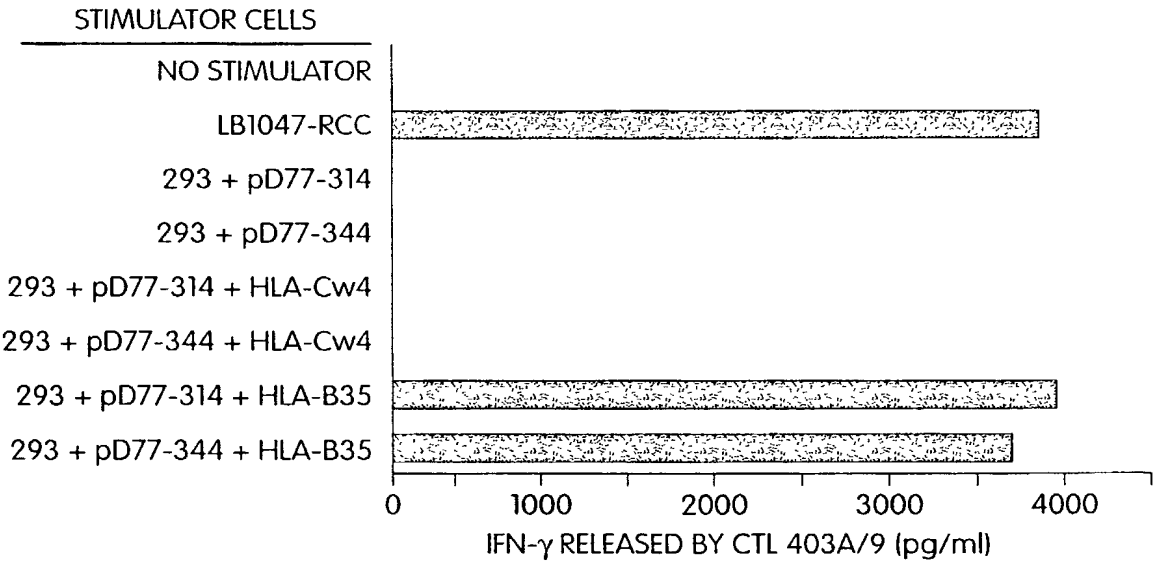
Fig. 2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293 + pD77-344 + HLA-B35
0
1000
2000
3000
4000
IFN-γ RELEASED BY CTL 403A/9 (pg/ml)

Fig. 3

MPO29/39 M A G L P A V V G L S P G E Q E Y H R G
alt.M-CSF M A G L P A V V G L S P G E Q E Y H R G G V G V L
ATG GCT GGG CTC CCT GCT GTT GTT GGT CTG TCT CCT GGC GAG CAG GAG TAT CAC CGA GGA GGT GTC GGA GTA CTG TAG
M-CSF M T A P G A A G R C P P T T W L G S L L L L V C L L A S R S I T E E V S E Y C S H M I ....
ATG ACC GCG CCG GGC GCC GCC GGG CGC TGC CCT CCC ACG ACA TGG CTG GGC TCC CTG CTG TTG TTG GTC TGT CTC CTG GCG AGC AGT ATC ACC GAG GAG GTG TCG GAG TAC TGT AGC CAC ATG ATT ....
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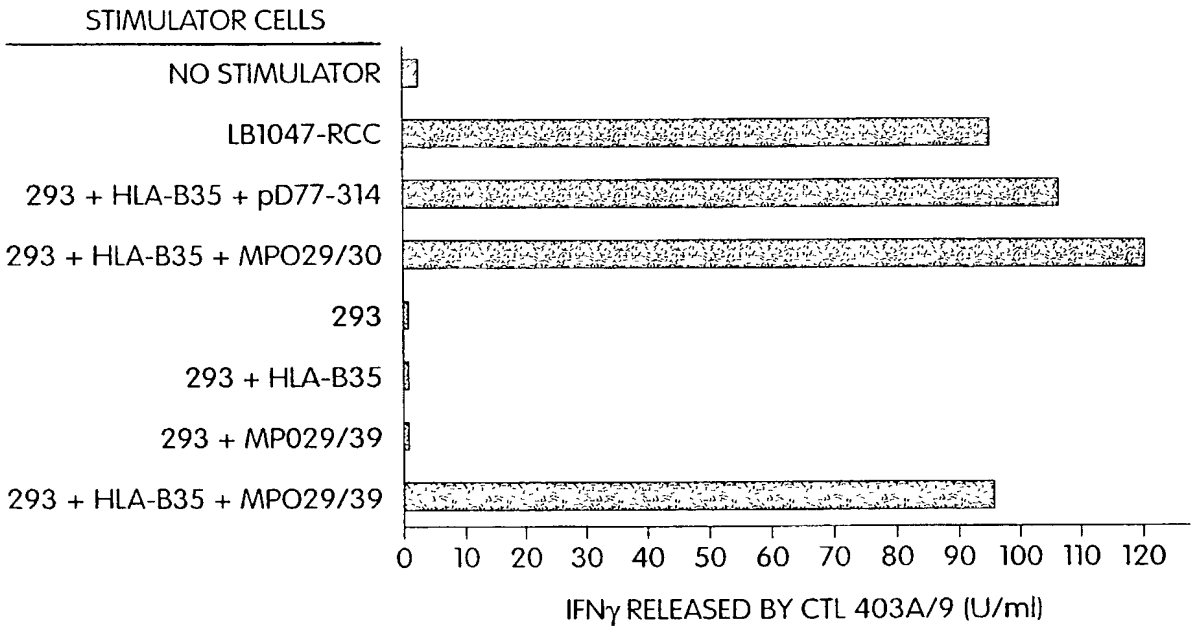
Fig. 4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293
293 + HLA-B35
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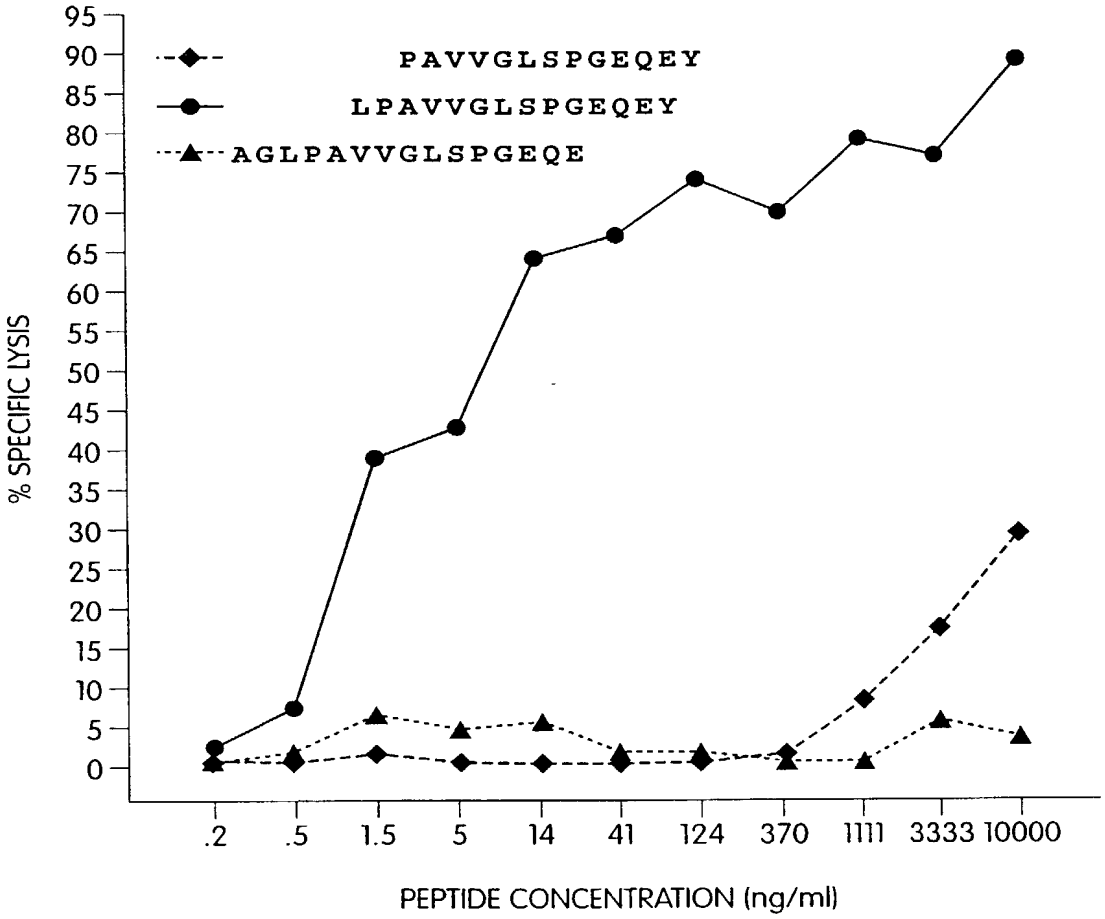
Fig. 5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75
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55
50
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40
35
30
25
20
15
10
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5
14
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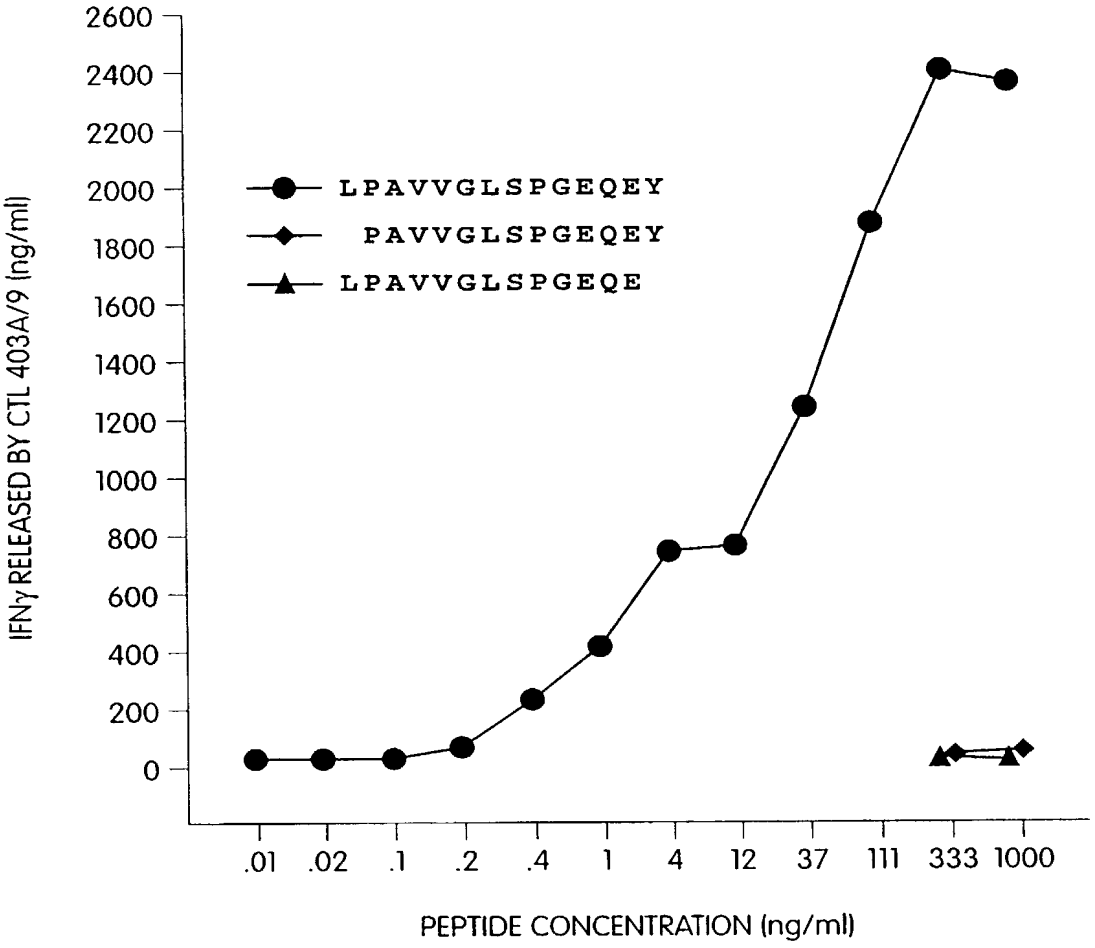
370
1111
3333
10000
PEPTIDE CONCENTRATION (ng/ml)

Fig. 6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2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
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LPAVVGLSPGEQEY
PAVVGLSPGEQEY
LPAVVGLSPGEQE
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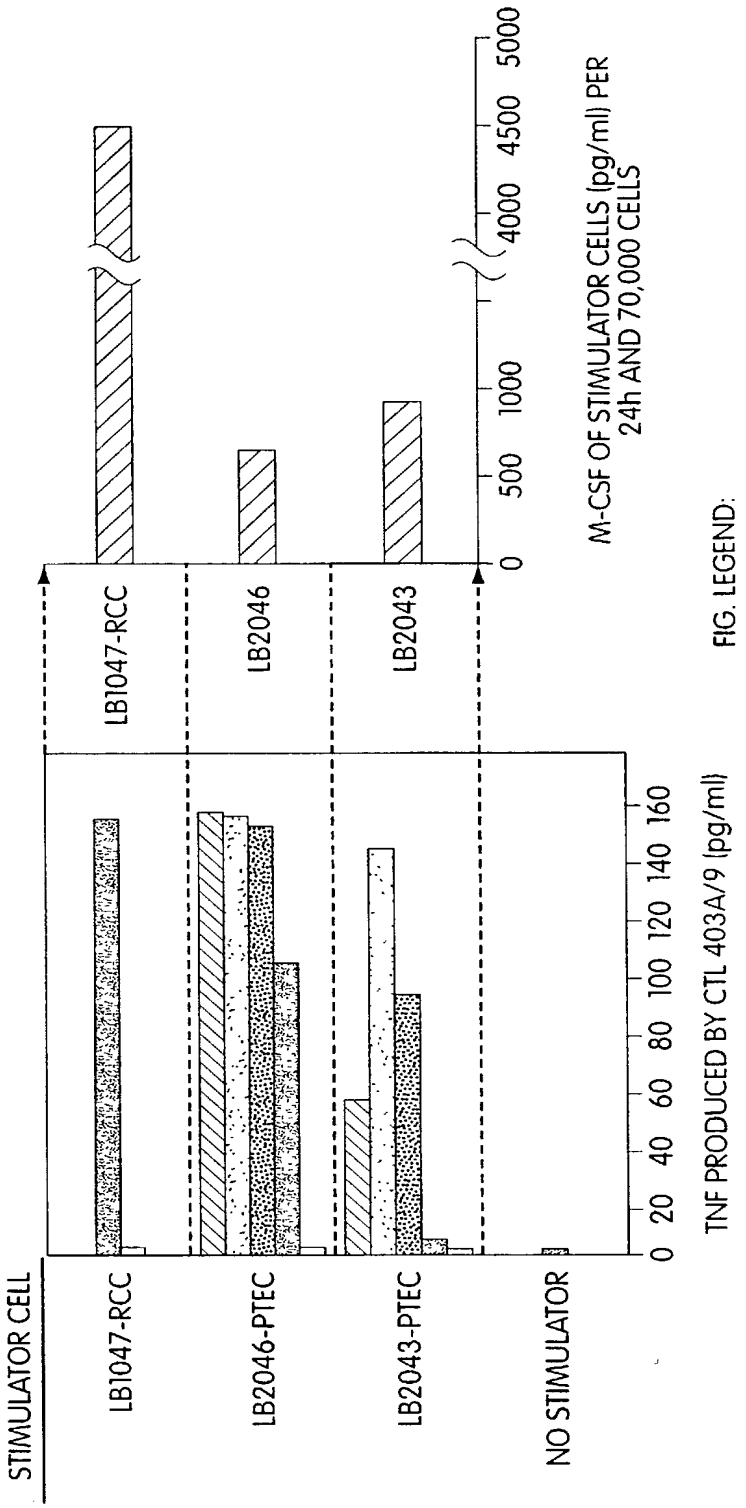
.02
.1
.2
.4
1
4
12
37
111
333
1000
PEPTIDE CONCENTRATION (ng/ml)

Fig. 7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LB2046-PTEC
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NO STIMULATOR
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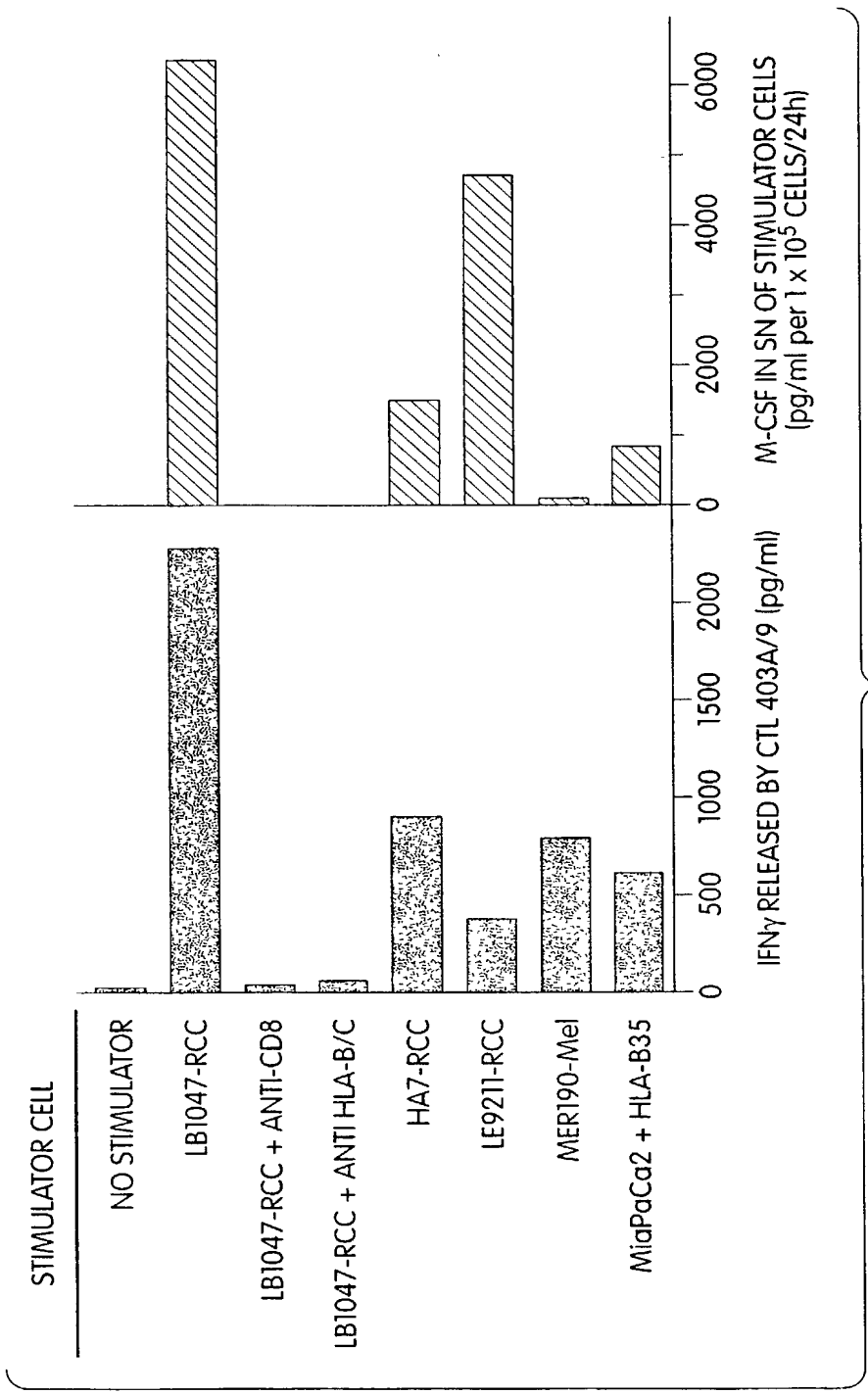
TNF PRODUCED BY CTL 403A/9 (pg/ml)
LB1047-RCC
LB2046
LB2043
0 500 1000 4000 4500 5000
M-CSF OF STIMULATOR CELLS (pg/ml) PER 24h AND 70,000 CELLS
FIG. LEGEND:
403A/9 + LPAVVGLSPGEQEY
403A/9 + HLA-B35 + LPAVVGLSPGEQEY
403A/9 + HLA-B35
403A/9
NO CTL

Fig. 8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LB1047-RCC
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LB1047-RCC + ANTI HLA-B/C
HA7-RCC
LE9211-RCC
MER190-Mel
MiaPaCa2 + HLA-B35
0
500
1000
1500
2000
IFNγ RELEASED BY CTL 403A/9 (pg/ml)
0
2000
4000
6000
M-CSF IN SN OF STIMULATOR CELLS (pg/ml per 1 x 10^5 CELLS/24h)

Fig. 9

Attorney Docket No. L0461/7102

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled

AN ANTIGENIC PEPTIDE ENCODED BY AN ALTERNATIVE OPEN READING FRAME OF HUMAN MACROPHAGE COLONY-STIMULATING FACTOR

the specification of which is attached hereto unless the following is checked:

[X] was filed on March 1, 2001, as United States Application No. 09/786,214, bearing attorney docket no. L0461/7102, and was amended on March 1, 2001.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or section 365(a) of any PCT International application designating at least one country other than the United States listed below and have also identified below any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed:

Prior Foreign PCT International Application(s)and any priority claims under 35 U.S.C. §§119 and 365(a),(b):

			Priority Claimed	
(Number)	(Country-if PCT, so indicate)	(DD/MM/YY Filed)	[ ] YES	[ ] NO
(Number)	(Country-if PCT, so indicate)	(DD/MM/YY Filed)	[ ] YES	[ ] NO
(Number)	(Country-if PCT, so indicate)	(DD/MM/YY Filed)	[ ] YES	[ ] NO

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below:

(Application Number)	(filing date)
(Application Number)	(filing date)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s), or §365(c) of any PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application No.)	(filing date)	(status-patented, pending, abandoned)
(Application No.)	(filing date)	(status-patented, pending, abandoned)

PCT International Applications designating the United States:

PCT/US9920344		3 September 1999	Patented
(PCT Appl. No.)	(U.S. Ser. No.)	(PCT filing date)	(status-patented,pending,abandoned)

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Robert M. Abrahamsen	40,886	Jason M. Honeyman	31,624	Edward J. Russavage	43,069
Eric Amundsen	46,518	Robert E. Hunt	39,231	Stanley Sacks	19,900
John N. Anastasi	37,765	Ronald J. Kransdorf	20,004	Christopher S. Schultz	37,929
Ilan Barzilay	46,540	Peter C. Lando	34,654	Alan B. Sherr	42,147
Gary S. Engelson	35,128	M. Brad Lawrence	47,210	Robert A. Skrivanek, Jr.	41,316
Neil P. Ferraro	39,188	Helen C. Lockhart	39,248	Alan W. Steele	45,128
Thomas G. Field, III	45,596	Matthew B. Lowrie	38,228	Mark Steinberg	40,829
Stephen R. Finch	42,534	William R. McClellan	29,409	Joseph Teja, Jr.	45,157
Edward R. Gates	31,616	Daniel P. McLoughlin	46,066	John R. Van Amsterdam	40,212
Richard F. Giunta	36,149	James H. Morris	34,681	Robert H. Walat	46,324
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William G. Gosz	27,787	Timothy J. Oyer	36,628	David Wolf	17,528
Lawrence M. Green	29,384	Edward F. Perlman	28,105	Douglas R. Wolf	36,971
George L. Greenfield	17,756	Michael J. Pomianek	46,190		
James M. Hanifin, Jr.	39,213	Elizabeth R. Plumer	36,637		
Therese A. Hendricks	30,389	Randy J. Pritzker	35,986		
Steven J. Henry	27,900	Robert E. Rigby, Jr.	36,904		

Address all telephone calls to John R. Van Amsterdam at telephone no. (617) 720-3500. Address all correspondence to:

John R. Van Amsterdam
c/o Wolf, Greenfield & Sacks, P.C.,
Federal Reserve Plaza
600 Atlantic Avenue
Boston, MA 02210-2211

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Michael Probst-Kepper 30.05.2001

Inventor's signature **Date**

1-00

Full name first inventor: **Michael Probst-Kepper**
Citizenship: Belgium
Residence: Brussels, Belgium BEX
Post Office Address: Avenue Hippocrate 74, UCL 7459, B-1200 Brussels, Belgium

Inventor's signature **Date**

Full name first inventor: **Benoit van den Eynde**
Citizenship: Belgium
Residence: Rixensart, Belgium
Post Office Address: Avenue Hippocrate 74, UCL 7459, B-1200 Brussels, Belgium

Inventor's signature **Date**

Full name first inventor: **Thierry Boon-Falleur**
Citizenship: Belgium
Residence: Brussels, Belgium
Post Office Address: Avenue Hippocrate 74, UCL 7459, B-1200 Brussels, Belgium

Attorney Docket No. L0461/7102

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Neil P. Ferraro	39,188	Helen C. Lockhart	39,248	Alan W. Steele	45,128
Thomas G. Field, III	45,596	Matthew B. Lowrie	38,228	Mark Steinberg	40,829
Stephen R. Finch	42,534	William R. McClellan	29,409	Joseph Teja, Jr.	45,157
Edward R. Gates	31,616	Daniel P. McLoughlin	46,066	John R. Van Amsterdam	40,212
Richard F. Giunta	36,149	James H. Morris	34,681	Robert H. Walat	46,324
Peter J. Gordon	35,164	M. Lawrence Oliverio	30,915	Lisa E. Winsor	44,405
William G. Gosz	27,787	Timothy J. Oyer	36,628	David Wolf	17,528
Lawrence M. Green	29,384	Edward F. Perlman	28,105	Douglas R. Wolf	36,971
George L. Greenfield	17,756	Michael J. Pomianek	46,190		
James M. Hanifin, Jr.	39,213	Elizabeth R. Plumer	36,637		
Therese A. Hendricks	30,389	Randy J. Pritzker	35,986		
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Inventor's signature **Date**

Full name first inventor: **Michael Probst-Kepper**
Citizenship: Belgium
Residence: Brussels, Belgium
Post Office Address: Avenue Hippocrate 74, UCL 7459, B-1200 Brussels, Belgium

2-00

Inventor's signature May 11 2001 **Date**

Full name first inventor: **Benoit van den Eynde**
Citizenship: Belgium
Residence: Rixensart, Belgium BEX
Post Office Address: Avenue Hippocrate 74, UCL 7459, B-1200 Brussels, Belgium

3-00

Inventor's signature May 15 2001 **Date**

Full name first inventor: **Thierry Boon-Falleur**
Citizenship: Belgium
Residence: Brussels, Belgium BEX
Post Office Address: Avenue Hippocrate 74, UCL 7459, B-1200 Brussels, Belgium

SEQUENCE LISTING

<110> Ludwig Institute for Cancer Research

<120> AN ANTIGENIC PEPTIDE ENCODED BY AN ALTERNATIVE OPEN READING FRAME OF HUMAN MACROPHAGE COLONY-STIMULATING FACTOR

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Gly Ser Leu Leu Leu Leu Val Cys Leu Leu Ala Ser Arg Ser Ile Thr
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gag gag gtg tcg gag tac tgt agc cac atg att                       129
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Glu Glu Val Ser Glu Tyr Cys Ser His Met Ile
        35                  40
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